A STUDY ON INVASIVE FUNGAL INFECTIONS AMONG IMMUNOCOMPROMISED PATIENTS IN A TERTIARY CARE HOSPITAL

Dissertation submitted to

THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY

in partial fulfillment of the regulations

for the award of the degree of

M.D. (MICROBIOLOGY)

BRANCH – IV

MADRAS MEDICAL COLLEGE,

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

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APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled **"A STUDY ON INVASIVE FUNGAL INFECTIONS AMONG IMMUNOCOMPROMISED PATIENTS IN A TERTIARY CARE HOSPITAL"** is a bonafide record of work done by **DR.K.S.KUMUDHAVALLI,** during the period of her post graduate study from May 2010 to April 2013 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai-600003, in partial fulfillment of the requirement for **M.D.MICROBIOLOGY** degree examination of The Tamilnadu Dr. M.G.R. Medical University to be held in April 2013.

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DECLARATION

I declare that the dissertation entitled **"A STUDY ON INVASIVE FUNGAL INFECTIONS AMONG IMMUNOCOMPROMISED PATIENTS IN A TERTIARY CARE HOSPITAL"** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***September 2011 to August 2012*** *under the guidance of Prof.* ***Dr.G.JAYALAKSHMI, M.D., D.T.C.D.,*** *Professor of Microbiology, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to the Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Microbiology (Branch IV) examination to be held in April 2013.*

Place: Chennai ***Signature of the Candidate***

Date :

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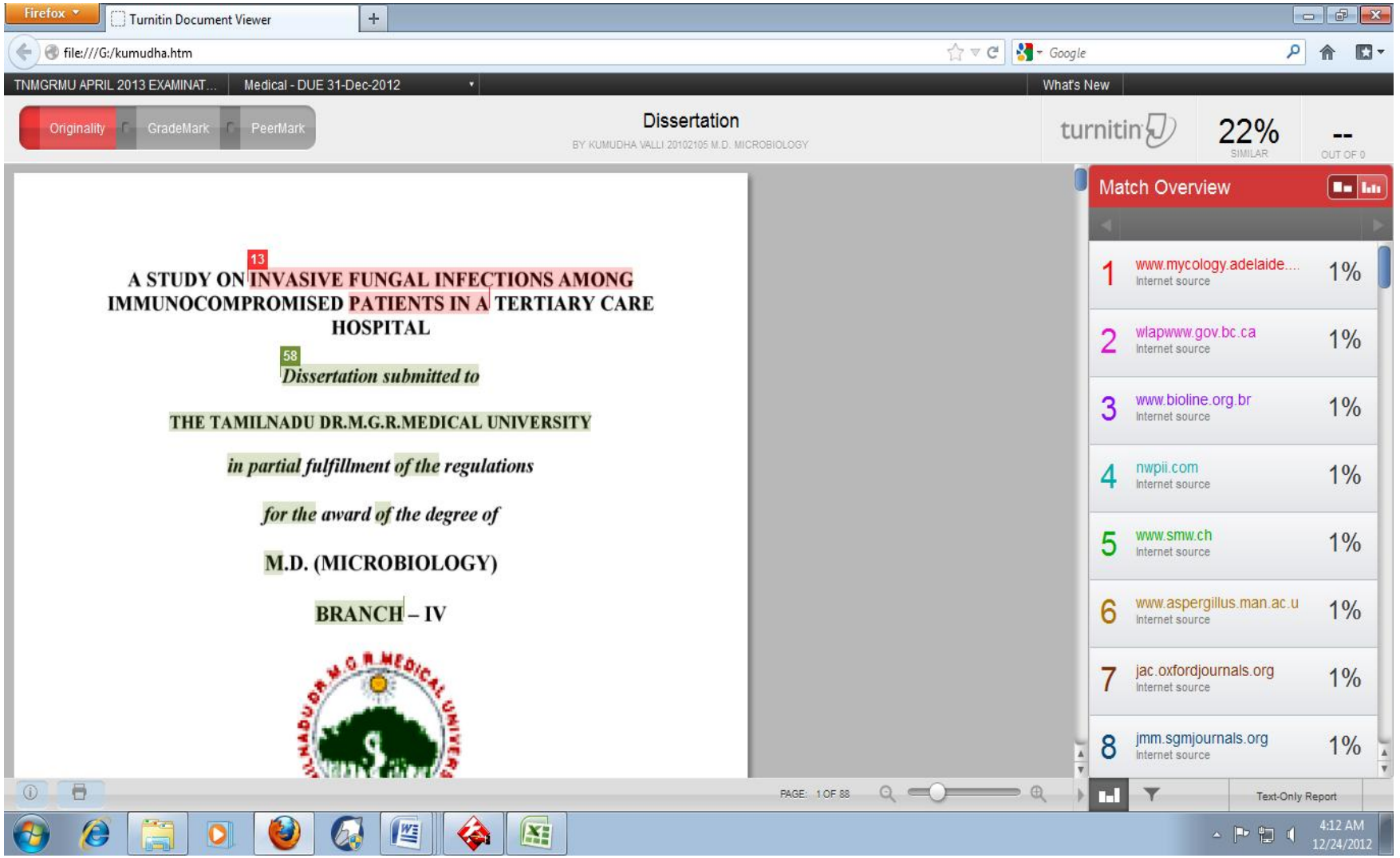
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INTRODUCTION

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India is a vast country with more than one billion people and spread over an area of 3.3 thousand million square kilometers [1,2]. The geographical and environmental conditions vary in different places of the country. Located in the tropics with heavy annual monsoon, the climatic conditions are favorable for the fungi to grow in many parts of the country. All of these factors together favour widespread occurrence of both common and unique mycotic infections. Invasive fungal infections (IFIs) have shown a steady rise in incidence among immunocompromised and critically ill patients in whom they are the major cause of morbidity and mortality [3,4]. The precise prevalence of disease is not known but population-based surveillance estimates it as 12–17 per 100 000 population [4,5,6].

Several reasons favoring IFIs include usage of anti neoplastic and immunosuppressive agents, broad-spectrum antibiotics, patients with uncontrolled diabetes mellitus, burns, neutropenia, HIV infection, prolonged intensive care unit (ICU) admission and aggressive surgery[7].

The frequency of IFI is 5-30% in patients with malignancy[3,8] , 2-42% in organ transplant recipients,20-25% among AIDS patients and 10-15% among Diabetes mellitus (DM) patients[3,9].

In India about 30million people are affected by diabetes mellitus and 3-6million people have HIV infection [10] . Solid organ transplantation are performed increasingly in tertiary care centers. Systemic steroids, immunosuppressive agents and chemotherapeutic agents are available all over the country for prevention of transplant rejection and for treatment of malignancy.

The diagnosis of IFI rests on a combination of clinical and laboratory parameters. Many of the suspected IFI are not proven. Slow growth of certain fungi such as dematiaceous fungi often causes delay in clinching the diagnosis. The exact incidence of opportunistic IFI is not known due to fewer diagnostic mycology laboratories. Most clinicians are unaware of manifestations of mycotic diseases. Only a handful of centers carry out routine medical autopsies. Thus, our knowledge about these diseases is just the tip of a huge iceberg [1].

Candida spp and *Aspergillus spp* are the most common etiological agents of invasive fungal infections[11-16]. The estimated annual incidence of invasive mycoses due to these pathogens is 72–228 infections per million population for *Candida* spp and 12–34 infections per million population for *Aspergillus* spp [17-23]. *Cryptococcus spp* is the most common isolate from AIDS patients[7] and those on corticosteriods in whom it causes pneumonia and meningitis with annual incidence of 30–66 infections per million population [17-23].

Zygomycetes are more common among patients with uncontrolled diabetes mellitus and those on iron therapy [24]. *Fusarium spp, Penicillium spp* and dematiaceous fungi are also reported among immunosuppressed patients [7].

Accurate diagnosis of invasive fungal infection remains a problem in immunocompromised patients, in whom signs and symptoms are nonspecific or develop late in the course of illness due to the defective immune response [4]. Thus, early diagnosis and prompt initiation of antifungal therapy are essential to reduce morbidity and mortality [11].

Antifungal drugs are the mainstay for management of IFI, which are in a continuum[11]. They are:

- Prophylaxis (administration of antifungal drugs to high-risk groups without evidence of disease)
- Empirical (administration of drugs to neutropenic patients with persistent refractory fever)
- Preemptive (antifungals used using clinical, radiological and laboratory markers to determine the likelihood of disease) and
- Treatment of established fungal infection.

Treatment may be prolonged for IFI and is often associated with drug toxicities. The high cost, limited availability, poor compliance are factors favouring emergence of antifungal resistance especially in the settings of immunosuppression [4].

As antifungal susceptibility testing is labour intensive and expensive it is not routinely performed in all laboratories. The treatment therefore rests on empirical antifungal therapy with no available data on antifungal susceptibility testing and no monitoring on emergence of antifungal resistance.

As few data are available in Indian literature on IFI, this study was undertaken. The etiological agents, the antifungal susceptibility pattern, the risk factors and categorization of IFI are studied.

AIM OF THE STUDY

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The present study was undertaken with the following aims and objectives.

- To isolate the fungi causing Invasive fungal infections
- To identify and speciate the fungi isolated
- To Identify the common fungal infections occurring in the study among patients with invasive fungal infections
- To categorize the type of Invasive fungal infections
- To study the morbid risk factor of Invasive fungal infections
- To assess the mortality rate among Invasive fungal infection patients
- To study the susceptibility pattern of the fungal isolates to standard anti fungal drugs
- To compare the different methods of antifungal susceptibility testing done for the fungal isolates

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Among the opportunistic infections, fungal infections have emerged as an important etiological agent. The fungal infections are now recognised as one of the significant causes of morbidity and mortality among the human beings. Invasive fungal infections were regarded as rare disease about a few decades ago. Since then there has been a steady increase in the number of patients suffering from life threatening fungal infections. Opportunistic fungal infection is a frequently encountered complication especially in immune compromised patients like patients suffering from Cancer [25,26], HIV, DM [27,28] etc. The overall incidence of invasive mycosis has been increased from 3% to 30% in of various institutions.

The systematic study of these organisms is about 150 years old. [29]In the past the fungi have caused only a small proportion of infectious disease, but with increasing control of infections due to bacteria and viruses through sanitation, education, immunization and chemotherapy, the fungal infections are becoming more important.[30]

The fungi are eukaryotic with a range of internal membrane system, membrane bound organelles and a well defined cell wall. They differ from bacteria in that they have multilayered cell wall containing chitin, mannan and other polysaccharides and peptides. The cytoplasm has sterols. The contents of the cytoplasm include mitochondria, endoplasmic reticulum etc. The nucleus has nuclear membrane and paired chromosomes and the mode of reproduction is both sexual and asexual resulting in spore formation[29].

In 1969, R.H. Whittaker gave an independent status to the fungi in a separate kingdom [31]. The current classification scheme follows that proposed by Whittaker *et al.* and modified by Morgulis and Schwartz *et al*. The classification of fungi is based primarily on the morphology, method of sexual and asexual reproduction [32].

Depending on the cell morphology they are classified into:

1. **Yeasts:** Unicellular fungi which occur mainly as single, spherical and ellipsoidal cells which reproduce by budding. eg., *Cryptococcus neoformans.*

2. **Yeast like fungi:** Grow partly as yeasts and partly as long filamentous cells joined end to end forming a pseudo mycelium. eg., *Candida albicans.*

3. **Moulds:** Grow as long filaments or hyphae which branch and interlace to form a meshwork or mycelium and reproduce by formation of various kinds of spores. eg., *Aspergillus spp, Mucor spp*, etc.

4. **Dimorphic fungi:** Grow either as filaments or yeasts according to the cultural conditions. Mycelial form of growth occurs on culture media at 22^0C and yeast form of growth occurs at 37^0C either on media or in animal body. eg., *Histoplasma capsulatum.*

The systematic classification of the fungi is based on the nature of their sexual reproduction [33].

1) **PHYCOMYCETS:** Form usually non septate hyphae and asexual sporangiospores contained within swollen spore case or sporangium borne at the ends of aerial hyphae.

2) **ASCOMYCETE:** Form septate hyphae and various kinds of asexual spores including conidia. Sexual ascospores are formed within a sac or ascus.

3) **BASIDIOMYCETES:** Form septate hyphae and sexual basidiospores usually four in number from the ends of the club shaped structure called basidia.

4) **FUNGI IMPERFECTI:** Include all those of which the sexual or perfect state has not been described and which therefore cannot be placed with certainly in one of the other three classes. A majority of pathogenic fungi belong to this group.

Very few fungi are capable of causing significant disease in otherwise normal individuals and the others are only able to produce disease under unusual circumstances mostly involving host debilitation. The incidence of fungal infections has increased to a great extent especially among the immune compromised individuals.

The term immune compromised host is defined as a person with impaired host defense mechanisms and who is at risk of developing an infection. The immune deficiency may be either as primary or congenital immune deficiency diseases such as severe combined immunodeficiency, thymic aplasia or other immunoglobulin deficiencies, and acquired immune deficiency disorders like AIDS, patients with autoimmune diseases being treated with corticosteroids or cytotoxic therapy, patients with diabetes mellitus, severe burns, prolonged antibiotic therapy etc. [34].

CATEGORISATION OF INVASIVE FUNGAL INFECTION

Standard definitions for IFIs was developed by European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group in 2008 into 3 levels[39] .

They are:

- Proven IFI
- Probable IFI
- Possible IFI

Three elements which form the basis for categorizing them are[39-41]:

- Host factors,
- Clinical manifestations
- Mycological results.

PROVEN INVASIVE FUNGAL INFECTIONS

<table>
<tr><td colspan="2">Deep tissue infections</td></tr>
<tr><td>Moulds

• Histo/cytochemistry and/or KOH showing hyphae from a biopsy or needle aspiration

OR

• Positive culture from a normally sterile site and clinically or radiologically abnormal site consistent with infection, obtained by a sterile procedure</td><td>Yeasts

• Histo/cytochemistry showing yeast cells and/or pseudohyphae from a biopsy or needle aspiration excluding mucous membranes

OR

• Positive culture obtained by a sterile procedure from a normally sterile site and clinically or radiologically abnormal site consistent with infection, excluding urine, sinuses, and mucous membranes

OR

• India ink or antigen positivity for Cryptococcus in CSF</td></tr>
<tr><td colspan="2">Fungemia</td></tr>
<tr><td>Moulds

• Positive blood culture of fungi excluding Aspergillus sp and Penicillium sp, other than P.marneffei, accompanied by clinical manifestations compatible with the relevant organism.</td><td>Yeasts

• Positive blood culture of Candida and other yeasts in patients with clinical manifestations compatible with the relevant organism.</td></tr>
</table>

PROBABLE INVASIVE FUNGAL INFECTIONS:

To define probable IFI, it needs atleast one criterion from each of the 3 elements.

- Host section
- Microbiological criterion
- One major (or two minor) clinical criteria

POSSIBLE INVASIVE FUNGAL INFECTIONS:

- One criterion from host section
- One microbiological OR one major (or two minor) clinical criteria from an abnormal site consistent with infection

ELEMENTS USED FOR DEFINING IFI:

Host factors

- Neutropenia (<500 neutrophils/mm^3 for >10 days)
- Persistent fever for >72 h refractory to appropriate treatment with broad-spectrum antibacterial drugs in high-risk patients
- Body temperature of >38^0C or <36^0C
- Recent usage of immunosuppressive agents for more than 30 days
- Previous episode of proven or probable invasive fungal infection in neutropenic patients

- AIDS patients
- Signs and symptoms indicating severe (grade 2) graft-versus-host disease
- Prolonged (>3 weeks) usage of corticosteroids for more than 60 days.

Microbiological criterion

- Positive culture for mould (including *Aspergillus, Fusarium,* or *Scedosporium spp* or Zygomycetes) or *Cryptococcus neoformans* from sputum or bronchoalveolar lavage fluid samples.
- Positive findings of cytologic/direct microscopic evaluation for mould or *Cryptococcus spp* from sputum or bronchoalveolar lavage fluid samples.
- Positive result for Cryptococcal antigen in blood sample.
- Positive findings of cytologic or direct microscopic examination for fungal elements in sterile body fluid samples (e.g., *Cryptococcus* species in CSF).
- Two positive reports of culture for yeasts from urine samples in absence of urinary catheter
- Positive result for *Candida* sp from blood culture.
- Pulmonary abnormality and negative bacterial cultures from specimen related to lower respiratory tract infection

CLINICAL CRITERIA

Lower Respiratory Tract Infection

Major

Any one of the following new infiltrates on CT imaging:

- halo sign,
- air crescent sign, or
- cavity within an area of consolidation

Minor

- Symptoms of LRTI (cough, chest pain, hemoptysis, dyspnea)
- Physical finding of pleural rub
- Any new infiltrate not fulfilling major criterion

Central Nervous System Infection

Major

Suggestive radiologic evidence of CNS infection (i.e. meningitis extending from a paranasal, auricular, or vertebral process;intracerebral abscesses or infarcts)

Minor

(CSF negative for pathogens by culture & microscopy)

1. Focal neurologic signs and symptoms
2. Mental changes
3. Meningeal irritation
4. Abnormalities in CSF biochemistry & cell count

EPIDEMIOLOGY OF INVASIVE FUNGAL INFECTIONS:

The fungi are widely found in the environment worldwide. Out of 1.5 million species of fungi estimated to exist all over the world, one third of them 27,000 species exist in India[42].

Invasive candidiasis

Candida spp is a normal commensal of skin, and gastrointestinal and genitourinary tracts. Candidiasis has no geographic limitation. Previously considered as commensals but now emerged as opportunistic pathogens causing severe infection and therapeutic problem.

Candida spp are the most frequent cause of fungal infections (70 to 90% of cases) and account for 5 to 15% of health-care associated infections[11,43,44]. Candidemia, one of the most frequent clinical manifestations of candidiasis, is associated with significant morbidity, prolonged hospital stay, high mortality of 40% to 60% and increased health care costs[45].

C. albicans was the predominant species to cause (40-60%) invasive candidiasis[11,46]. However, a shift towards non- *Candida albicans* (NCA) species with *C.tropicalis* (25%), *C.glabrata* (8%),*C.parapsilosis*(7%),*C.krusei*(4%) are reported . They are of special concern due to high virulence nature and reduced susceptibility to antifungal agents, so identification to species level and susceptibility testing are recommended that all fungi obtained from sterile sites and urine of immunocompromised patients [47] .

Candidemia rates increased rapidly since 1980s and *Candida spp* was the fourth-commonest cause of bloodstream infection (BSI) [48] . However in the late 1990s

C. albicans incidence as BSI was decreased[49] but the incidence of NCA BSI mostly due to *Candida glabrata* remained stable. Prior surgery, acute renal failure, neutropenia, antibacterial agents, parenteral nutrition and central venous catheters were associated with an increased risk of invasive candidiasis.[50]

Cryptococcosis[24]:

Cryptococcosis is also known as torulosis or European Blastomycosis. The prevalence of cryptococcosis varies from 3- 6% in Europe, 6-10% in USA and 10-30% in Central Africa. Cryptococcosis caused by the capsulated yeast *Cryptococcus neoformans*, is principally a disease of the central nervous system, although the primary site of infection is the lungs(30-40%). Almost all cases are caused by *C. neoformans* var. *neoformans.*[51-53]

Cryptococcal meningitis occurs most frequently in patients with abnormal T lymphocyte function. These include patients with AIDS, Hodgkin's disease, collagen disease and neoplasms.

A mild, self-limiting pulmonary infection is believed to be the commonest form of cryptococcosis which represents the primary infection. In a proportion of patients, the disease will progress to a chronic meningitis or meningoencephalitis.

Cryptococcus neoformans var grubii causes manifestation in HIV positive patients where as Cryptococcus neoformans var neoformans are more common among patients on corticosteroids[42].AIDS patients with Cryptococcosis mostly develop chronic meningeal form and the symptoms tend to be milder than in other patients. Cryptococcosis in AIDS patients is considered incurable and needs lifelong therapy to

suppress the infection. A positive serum Cryptococcal antigen is reliable for the diagnosis of disseminated disease.[47,54].

Invasive Aspergillosis

Invasive Aspergillosis (IA) is the second most common cause for invasive mycosis[11,55] accounting for 10 to 20% of all cases[11]. *Aspergillus spp.* can lead to invasive aspergillosis, tracheobronchitis, aspergilloma and chronic necrotizing aspergillosis mainly in immmunocompromised hosts, including neutropenic (particularly if >3 weeks) cancer patients, patients treated with immunosuppressive therapies (corticosteroids, cytotoxic agents or monoclonal antibodies acting as anti-immune mediators) [7] and in AIDS patients. IA was also reported in <1% of renal transplant recipients [56] .

The most frequent site of IA is the lower respiratory tract which accounts for about 80 to 90%[11]. Other clinical manifestations include sinusitis, central nervous system (CNS) infections and disseminated infection involving multiple sites [7, 57].

A.fumigatus is the predominant *Aspergillus spp* isolated from IA accounting for more than 90%, followed by *A. flavus, A. terreus* and *A. niger*[11]. However, a shift in the epidemiology of *Aspergillus* infections by non-*fumigatus Aspergillus* species, such as *A. terreus* which is resistant to amphotericin B are seen.

Accurate diagnosis of invasive infection, particularly aspergillosis, remains problematic in patients with haematological malignancy, in whom signs and symptoms are nonspecific and often develop late in the course of infection. Mortality from aspergillosis is 70% if diagnosis is delayed. [10]

EMERGING MOULDS:

Fusarium spp is a filamentous fungus widely distributed on plants, in soil and in water[58-60]. Currently, there are over 50 known species of *Fusarium,* and only some causing infections in humans[61]. Notably, *Fusarium spp* is an emerging cause of opportunistic mycoses. *Fusarium* enters the host most commonly through the airways, via inhalation of aerosolized conidia. Nosocomial infections have been reported and may be caused by contamination of hospital water systems or hospital plants. Most commonly, *Fusarium spp* affects cancer patients, particularly those with acute leukemia, but can affect other immunocompromised hosts[17,62,63].

Zygomycosis[24] is a disease caused by members of the Mucorales. The disease classically involves the rhino-facial-cranial area, lungs, gastrointestinal tract, or less commonly other organ systems. The disease is associated with the diabetic keto acidosis,severely burned patients and other diseases such as leukemia and lymphoma, immunosuppressive therapy and corticosteroids. The fungi show a predilection for vessel (arterial) invasion resulting in embolization and necrosis of surrounding tissue. Infections are typically acute and fulminant. Rhinocerebral disease in acidotic patients usually results in death, often within a few days and the prognosis is grave[24].

Other moulds like *Penicillium spp* ,*Scedosporium spp*, (hyalohyphomycoses) , and dematatious fungi(Phaeohyphomycosis)[46] are also capable of causing a wide variety of IFI in immunocompromised host.[64].

RISK FACTORS FOR INVASIVE FUNGAL INFECTIONS[46]

- **Acquired-** infection such as HIV, prior invasive fungal infections and certain cancers including leukemia, lymphoma, or multiple myeloma,

Central Venous or urinary catheter, Prolonged stay in ICU, major surgery, burns etc

- **Chronic diseases** - such as end stage renal disease and dialysis, diabetes, cirrhosis
- **Medications** - such as steroids, chemotherapy, radiation, immunosuppressive post-transplant medications

FUNGAL PATHOGENICITY

The mechanism of fungal pathogenicity depends on its ability to adapt to the tissue environment of the host and to withstand the lytic activity of the host's defenses[51].The ability of fungi to invade tissue to cause human disease are primarily related to the immunological status of the host and environmental exposure. [35,51,65,66].

Most fungi are unable to grow at 37°C. Hence, thermo tolerance is essential to survive at this temperature for the fungi to grow within human body. Most fungi are saprophytic and their enzymatic pathways functions better at the redox potential of non-living substrates than at the lowered oxidation-reduction state of living tissue. However, many fungi prove to be able to surpass these two major physiologic barriers[51] .

Host defenses are of non-specific and specific nature.

NON-SPECIFIC DEFENSE MECHANISM :

- ✓ Antifungal activity of natural excretions, such as saliva and sweat;

- ✓ Protective effects of the endogenous normal micro biota of the skin and mucous membranes
- ✓ Mechanical barrier of the skin and mucous membranes preventing entry of fungi.
- ✓ Presence of highly efficient non-specific inflammatory system(neutrophils, mono- nuclear phagocytes and other granulocytes) to combat fungal proliferation

SPECIFIC DEFENCE MECHANISM: by acquired immunity defending from fungal growth in tissue consist basically of

- ✓ Cell mediated immunity regulated by T-lymphocytes
- ✓ Humoral immunity regulated by B-lymphocytes

Predisposing factors associated to defects in the host defenses against fungi[35,51]:

1) Disturbance in the epithelial barrier by:

- Broad spectrum or multiple antibiotic therapy
- Indwelling catheters
- Peritoneal dialysis
- Burns, ulcers, trauma, surgeries etc
- Increased gastric pH, cytotoxins, radiotherapy

2) Defects or dysfunction of mononuclear phagocytes, neutrophils and other cells caused by:

- Chemotherapy, radiotherapy
- Aplastic anaemia
- Chronic granulomatous disease
- Diabetes mellitus

3) Defect or dysfunction of T-lymphocyte resulting in poor cell mediated immunity caused by:

- AIDS
- Hodgkin's disease and Non hodgkins disease
- Post transplant patients on immunosuppressants
- Chemotherapy and radiotherapy
- Leukemia and Carcinoma
- Corticosteroids

SPECIMEN COLLECTION AND PROCESSING:

The laboratory diagnosis starts with collection of appropriate specimen. The anatomical site in which the organism is present must be carefully selected and the specimen collected in such a manner that it will allow the fungus to remain viable in its 'natural' state with no contamination. The collection, transport and processing of clinical specimens encompass one of the most important consideration in determining the etiology of fungal disease. Under strict aseptic precautions samples are collected

from the patients and transported immediately to the laboratory in appropriate settings and sample processing done.

SPECIMENS:

The following fungal species were most commonly recovered from the clinical specimens:[67]

Blood	**Pus and other exudates**	**Respiratory secretions**
• *Candida spp* • *Cryptococcus neoformans* • *Histoplasma capsulatum* • filamentous fungi are rarely isolated from blood with exception of *Fusarium spp*	• *Cryptococcus neoformans* • Dimorphic fungi • *Fusarium spp*	• *Aspergillus spp* • *Candida spp* • *Cryptococcus neoformans* • Dimorphic fungi • *Mucor spp* •*Scedosporium apiospermum* • *Rhizopus spp*

Miscellaneous body fluids			
Cerebrospinal fluid	**Urine**	**Vitreous Fluid**	**Synovial Fluid**
•*Cryptococcus neoformans* • *Candida spp* • *Coccidioides immitis* • *Histoplasma capsulatum*	• *Candida spp* • *Cryptococcus neoformans*	• *Candida albicans* (most common)	• *Aspergillus spp* • *Candida spp*

CLINICAL FEATURES:

Clinical symptoms of IFI are often non-specific, therefore a high index of suspicion in high risk patients is necessary for timely diagnosis and prompt treatment. Diagnosis of the IFI could be challenging, particularly in severely immunocompromised patients as a result of blunted inflammatory response due to profound neutropenia and altered T-cell function; hence, in such patients clinical and radiologic findings may be very subtle.

The most common clinical signs are the symptoms of local or generalized inflammatory response, primarily persistent fever despite the use of broad spectrum antibiotics. Other symptoms significantly depend upon the involved anatomic site and causative pathogen.

Diagnosis of systemic fungal infection based on clinical features and/or laboratory parameters : [68]

Esophagitis

- Endoscopically visualized plaques in the esophagus are clinically suggestive of fungal infection
- Positive fungal culture
- Pseudohyphae on Gram stain or on biopsy demonstrate invasive fungal elements

Pneumonia

FEATURES OF ASPERGILLUS, PSEUDALLESCHERIA, AND FUSARIUM PNEUMONIA:

- Persistent or progressive pulmonary infiltrate refractory to antibacterial therapy
- Clinical symptoms of pneumonia (fever, cough, haemoptysis, dyspnea, pleuritic pain, rales, and bronchial or pleural rub)
- Characteristic chest X-ray or CT findings:

- Subpleural radiologic densities, nodules, and wedge-shaped or cavitating lesions
- 'Air crescent' or 'halo sign' on CT scan
- Progression of lesions from infiltrates to cavity or crescent lesions

Urinary tract infection

- Signs and symptoms of bladder irritation (dysuria, hematuria, frequency, urgency, and suprapubic tenderness)
- Clean catch midstream or catheterized urine sediment containing 10^4 cfu/ml of *Candida spp*.

Fungemia

- At least one positive blood culture yielding fungus during a febrile episode
- Persistent *Candida* antigenemia

Abscess from deep sites(Brain abscess):

- Radiographic evidence of inflammatory focus
- Positive fungal culture from biopsy or aspiration

Endophthalmitis

- Ophthalmoscopic examination suggestive of endophthalmitis
- Positive fungal culture from either the eye, blood, or other sites of dissemination

INVESTIGATION

To improve outcomes of high risk patients it is critical to establish the diagnosis of IFI early, but currently there is no single diagnostic method that has a sufficient sensitivity and specificity to determine IFI. Therefore, timely diagnosis of IFI should be made on the basis of a constellation of clinical signs, confirmatory imaging studies and laboratory findings.

- Complete Blood count: Haemoglobulin %, ESR, Total count, Differential count, Platelet count
- Blood sugar level

- Liver function test
- Renal function test
- HIV testing and CD4 count

IMAGING STUDIES:

(i) PLAIN RADIOGRAPHY: Even minor abnormalities on chest radiographs (CXR) in high risk patients should prompt further investigation, which often includes computed tomography (CT) of the chest. CXR may be negative in up to 10% cases of invasive pulmonary Aspergillosis.

(ii) COMPUTED TOMOGRAPHY: When suspicion for invasive pulmonary Aspergillosis is high, it is very important to pursue CT imaging of chest[69].

CT findings that are more specific for IA are:

- "**Halo sign**" as a early finding when the central nodular area of fungal invasion is surrounded by a ground-glass appearing hemorrhage
- "**Crescent sign**" occur later as a result of necrosis and cavitation of lung tissue

(iii) MAGNETIC RESONANCE IMAGING: MRI is recommended for infections impending into orbit and intracranial compartment.

(iv) ENDOSCOPY: Upper gastrointestinal(UGI) endoscopy for invasive esophageal candidiasis and cystoscopy for bladder IFI.

MICROBIOLOGICAL LABORATORY DIAGNOSIS:

DIRECT EXAMINATION: [42]

WET MOUNT: Yeast cells and hyphal elements of the organism can be readily observed in routine 10% Potassium hydroxide preparations with or without fluorescent compound such as calcoflour white are used. For CSF samples, India ink or 10% Nigrosin negative stain preparation performed.

STAINING: Identifications are also aided by fungal stains such as Gomori methenamine silver, Periodic Acid Schiff in tissue. Zygomycetes stain poorly by Periodic Acid Schiff stain but stains better with H&E and Gomori methenamine silver.

As most of the organisms are common lab contaminants, microscopic demonstration of the presence of them in clinical material taken from lesion is significant.

CULTURE:

Non selective types of culture media are essential for primary isolation of fungi from clinical specimens. A combination of media is always better because it broadens the chances of isolating etiological agent. Various t types of culture media available for fungus isolation are[42]:

A. BASAL MEDIA:

1) SABOURAUD DEXTROSE AGAR:

SAB agar is the standard medium for recovery and maintenance of a wide variety of fungi commonly isolated in the clinical laboratory

because it provides basic nutrients that supports the growth of almost all fungi.

B. NUTRITIONALLY DEFICIENT MEDIA:

1) CORN MEAL AGAR(CMA):

Mostly used for chlamydospore production in yeast isolates.

2) RICE STARCH AGAR:

Used for chlamydospore formation faster than CMA in yeast isolates

C. **ENRICHED AND SELECTIVE MEDIA**:

1) BRAIN HEART INFUSION AGAR:

BHI is an enriched medium that enhances the recovery of *Cryptococcus neoformans* and fastidious pathogenic fungi from sterile specimens such as CSF

2) BIPHASIC MEDIUM:

This medium is used to isolate fungi from blood cultures.

3) SHEEP BLOOD AGAR:

Used for isolation of *Cryptococcus spp.*

4) CZAPEK-DOX AGAR:

This medium is useful for isolation of *Aspergillus* and *Penicillium spp*.

5) MALT EXTRACT AGAR:

This medium is used for maintenance of fungi in mycology laboratory. It is also an effective alternate media for the isolation of zygomycetes.

6) POTATO DEXTROSE AGAR:

This medium is used for stimulation of sporulation and pigment production.

ANTIGEN DETECTION

In immunocompromised patients with invasive disease who cannot mount good immune response, antigen detection may be very useful. Several tests are available for detection of soluble antigen of *Candida spp, Cryptococcus spp* and *Aspergillus spp* in serum, urine or other body fluids. Radio immune assay, Enzyme linked immunesorbant assay, Latex agglutination and Limulus lysate assay are most commonly used methods. Commercially available kits are Latex agglutination for *Cryptococcus spp,* G or Glucan test for *Candida spp* and *Aspergillus spp* and Galactomannan test for *Aspergillus spp*.These tests helps in earliest diagnosis of Invasive infection[42].

MOLECULAR DIAGNOSIS:

(i) NUCLEIC ACID DETECTION TECHNIQUES: Though highly sensitive and specific, they are still in experimental stage. Polymerase chain reaction (PCR), Nested PCR, Real time PCR, Branched DNA Technology though helps in detection and quantification of fungal DNA diagnosis in clinical specimens but cannot differentiate whether the organism is pathogen or colonizer[70].

(ii) TYPING OR SEQUENSING SYSTEMS: It is done mainly for epidemiological studies. Pulsed- Field Gel Electrophoresis, Restriction fragment length polymorphism analysis, Random amplified polymorphic DNA analysis are methods available[71].

Overview of Fungal Diagnostic Techniques[46]

Method	Pathogen(s) Detected	Comments
Traditional Methods		
Culture	All	Replication time for fungi is longer than bacteria and takes a longer time to complete; may be negative for certain fungal pathogens; unable to differentiate colonization from true pathogen; requires invasive specimen
Histopathology	All	Cannot confirm identification because many pathogens are morphologically similar; requires invasive specimen
Radiology	All	Cannot identify specific pathogen and difficult to distinguish from bacterial or other causes; lack of response in immunosuppressed patients results in false-negative results
Rapid Diagnostic Tools		
Galactomannan	*Aspergillus* only	False positive occurs with β-lactam antibiotics; low sensitivity in solid-organ transplant recipients; controversy exits regarding positive test cutoff[a]
Beta-glucan	*Candida* spp. and *Aspergillus* only	False positive occurs with dialysis filters, albumin, immune globulin; controversy exits regarding positive test cutoff[b]
Fungal PCR	All test is specific to organism	Commercially not available
PNA FISH	*Candida albicans* and *Candida glabrata*	

(i) [a]Controversy exists regarding cutoff for a positive test should be greater than 0.5 or 1.

(ii) [b]Controversy exists regarding cutoff for a positive test should be 60 pg/mL or 80 pg/mL.

ANTIFUNGAL SUSCEPTIBILITY TEST (AST)[42]:

The antifungal susceptibility testing is performed to provide information to clinician to select an appropriate antifungal agent for a treating an infection and to detect susceptibility pattern of the strain.There are various AST methods available, they are:

I. **CLSI METHODS**: Clinical and laboratory standards institute at Wayne, Pennsylvania deals with the susceptibility testing of various microorganisms including fungi. The approved version of CLSI document M27-A2(1997) and M38-A(1998) on standardized broth macro and micro broth dilution methods for AST of yeasts and moulds respectively.

Critical facts affecting reproducibility of AST:

- Inoculum size and preparation
- Incubation time & temperature
- Media used
- End point criteria
- Stability of antifungal agent
- Growth rate of fungi

BREAK POINT: Break point values detects the success or failure of an antifungal agent.

MEASUREMENTS: the final reading is taken either by turbidity measurement or colorimetric methods.

II. **AGAR DILUTION METHOD:** The aim of agar dilution method is to determine the MIC, under defined test conditions which inhibits the visible growth of the fungi being investigated. It involves incorporation of different concentrations of the antimicrobial substance in yeast nitrogen base agar medium followed by the application of a standardized number of cells on the surface of the agar plate [72].

III. **E TEST METHOD**: It is based on a combination of dilution and diffusion test principle and determines MIC. It is similar to disc diffusion method where, the discs are replaced by a thin, inert and non-porous calibrated plastic strip (5 x 60 mm) impregnated with a stable concentration gradient(corresponding to 15- two fold dilution) of antifungal compound. The results of E test and broth method were promising for *Candida spp* but marked disparity was seen among *Cryptococcus spp.*

IV. **DISC DIFFUSION METHOD**: The CLSI document for this method is M44-A. Disc containing antifungal agent placed on the surface of agar, diffuses into the surrounding medium, inhibiting the growth of the fungi and measurements of zone of inhibition are taken accordingly. Results are interpreted as:

- Susceptible: The zone diameter of test strain is more than 80% of control strain

- Intermediate or Susceptible dose dependent: The zone diameter of is less than 80% of control strain
- Resistant : There is no zone of inhibition

V. **FUNGITEST**: It is a microbroth dilution method based on CLSI M27-A2 standards. Test plate consists of disposable microplate with serial dilutions dehydrated drugs & colorimetric indicator and they are reconstituted by adding inoculums suspended in RPMI medium. It is an alternative to CLSI reference procedure[75].

VI. **SPECTROPHOTOMETRIC METHOD**: MIC is determined by reading broth microdilution plates with yellow coloured tetrazolium salts as indicator, which penetrates rapidly into the intact cells and converted to purple coloured formazan in spectrophotometer.

VII. **FLOW CYTOMETRY**: provides a rapid (4-6hrs) assay of antifungal activity by detecting the accumulation of vital dye in drug damaged fungal cells.

VIII. **OTHERS**: BacT/Alert can be tried for AST.

TREATMENT:

I. Surgical therapy:

Debridement of grossly infected and devitalized tissue is mandatory if lesion is localized. They have been tried for deep abscesses and lung infections.

II. Antifungal therapy:

There are four common antifungal strategies used in current clinical practice to combat IFI. These include antifungal prophylaxis, empirical therapy, pre-emptive (or diagnostics driven) therapy and treatment of proven and probable IFI.

Clinically useful antifungals are:

- Polyenes: Amphotericin B, Amphotericin B lipid formulation
- Azoles: Fluconazole,Itraconazole,Voriconazole,Posaconazole
- Echinocandins: Caspofungin,Micafungin,Anidulafungin
- Miscellaneous: Flucytosine

AMPHOTERICIN B:[7,11,76]

Mechanism of action: it binds to ergosterol in fungal cytoplasmic membrane, increasing permeability and causing leakage of intracellular components.

Spectrum of activity: good activity against most *Candida spp, Aspergillus spp*, *Cryptococcus spp* and dimorphic moulds.

LIPID FORMULATIONS OF AMPHOTERICIN B:

- Amphotericin B colloidal dispersion(ABCD)
- Amphotericin B lipid complex(ABLC)
- Liposomal Amphotericin B

Dosage:

Dose/Route	Amphotericin B	ABCD	ABLC	Liposomal Amphotericin B
mg/kg/iv	0.5-1	1.5	5	3

AZOLES[7,11,19-21,77-79]:

Mechanism of action: inhibits cytochrome P-450 dependent lanosterol 14-demethylase, an enzyme needed for synthesis of ergosterol, which form the main component of fungal cell membrane. This results in accumulation of methylated sterols, depletion of ergosterol and inhibition of cell growth.

Spectrum of activity:

Pattern of susceptibility of Yeast and Yeast like isolates for various azoles:

Organisms	Fluconazole	Itraconazole	Voriconazole	Posaconazole
Cryptococcus neoformans	S	-	S	R
Candida albicans	S	S	S	S
Candida tropicalis	S	S	S	S
Candida parapsilosis	S	S	S	S
Candida glabrata	SDD to R	SDD to R	S to I	S
Candida krusei	R	SDD to R	S	S
Candida lusitaniae	S	S	S	S

S: Susceptible; SDD: Susceptible dose dependent; I: Intermediate; R: Resistant;

Pattern of susceptibility of filamentous fungi isolates for various azoles:

Organisms	Itraconazole	Voriconazole	Posaconazole
A.fumigatus	+	++	++
A.flavus	++	++	++
A.terreus	+/-	+	++
A.nidulans	++	++	+
Penicillium spp	++	++	+
Fusarium spp	-	-	-/+
Rhizopus spp	-/+	-	++
Mucor spp	-/+	-	+
Dematiaceous fungi	+/++	+/++	+/++
Dimorphic fungi	++	++	-

Dosage	Fluconazole	Itraconazole	Voriconazole	Posaconazole
	200mg b.i.d	200mg b.i.d	200mg q12h Or 6mg/kg q12h IV	100mg b.i.d

ECHINOCANDINS[7,11,89]:

Mechanism of action: Non competitive inhibition of enzyme glucan synthase which produces (1,3)β d glucan. Destruction of cell wall leads to osmotic instability and lysis of fungal cell

Types:

- Caspofungin: Candidiasis in febrile neutropenic patients
- Micafungin: Invasive/ Esophageal Candidiasis

- Anidulafungin: Candidiasis in non neutropenic patients

Spectrum of activity of Caspofungin:

Potent fungicidal activity was seen against *Candida spp* and variable activity against *Aspergillus spp* and Dimorphic fungi.

Dosage: 70mg iv loading dose followed by a daily 50mg iv dose

FLUCYTOSINE: (5-FLUOROCYTOSINE) – 5-FC pyrimidine anologue[91]

Mechanism of action: By fungal cytosine deaminase enzyme ,5-FC is converted to an antimetabolite 5- Flurouracil which inhibits thymidylate synthetase needed for synthesis of DNA. So it acts by inhibiting DNA synthesis.

Spectrum of activity: has only narrow spectrum activity (action against *Candida spp*, *Cryptococcus neoformans* and few pheoid fungi.

Dosage:

- oral tablets - 25 mg/kg at 6 h intervals
- parenteral administration- 10 mg/ml in saline solution.

MATERIALS AND METHODS

MATERIALS AND METHODS

STUDY PLACE:

This cross sectional study was conducted in the Institute of Microbiology in association with the Institute of Dialectology, Department of Nephrology, Department of Haematology and Department of Oncology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai-3.

STUDY PERIOD:

The study was undertaken for a period of one year from September 2011 to August 2012.

ETHICAL CONSIDERATIONS:

Approval for the study was obtained from the Institutional Ethical Committee, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai-3 before commencing it. Informed consent was obtained from the study population. All patients satisfying the inclusion criteria were documented. Patients were interviewed by structured questionnaire.

STUDY POPULATION:

Inclusion criteria:

All adults more than 18 years of age with following clinical conditions were included:

- Acquired Immunodeficiency syndrome patients.

- Renal transplant patients on Immunosuppressive therapy for >30days
- Diabetic mellitus patients.
- Carcinoma, Leukemia and Lymphoma patients on chemotherapy for >30 days

Exclusion criteria:

- Immunocompromised conditions due to various other reasons.
- Chronic diseases such as cirrhosis, tuberculosis, end stage renal disease
- Certain conditions like Chronic granulomatous disease, Collagen vascular disease patients on steroid therapy.
- Burns patients & patients on dialysis

DATA COLLECTION:

Data collection included name, age, sex, address, IP number, date of admission, diagnosis at admission, physical examination findings and demographic profile which includes H/O Diabetes mellitus, Chronic kidney disease, neoplasm, immunosuppressive therapy, previous IFI.

CASE DEFINITIONS:[41]

Invasive fungal infections are defined in terms of “PROVEN”, “PROBABLE”, “POSSIBLE”,

PROVEN IFI:

Moulds

- Histo/cytochemistry and / or KOH showing hyphae from a needle aspiration or biopsy
- Positive culture obtained by sterile procedure from a normally sterile site and clinically and radiologically abnormal site consistent with infection.
- Positive blood culture of fungi excluding *Aspergillus spp* and *Penicillium* spp other than *P.marneffei*, accompanied with clinical manifestations.

Yeasts

- Histo/cytochemistry showing yeast cells and/or pseudohyphae from a biopsy or needle aspiration excluding mucous membranes
- Positive culture obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with infection excluding urine, sinuses, and mucous membranes
- India ink or antigen positivity for *Cryptococcus spp* in CSF
- Positive blood culture of *Candida spp.*

PROBABLE IFI:

- Atleast one criteria from host factor, one microbiological criteria **and** one major (or two minor) clinical criteria from abnormal site consistent with infection.

POSSIBLE IFI:

- Atleast one criteria from host factor and one microbiological criteria **or** one major (or two minor) clinical criteria from abnormal site consistent with infection.

SAMPLE COLLECTION:

Sample collection was done according to American Thoracic Society Recommendations for fungal culture[82]

SPECIMEN	**RECOMMENDED PROCEDURE**
Sputum	First early morning sample collected before breakfast after vigorous rinsing of mouth with water. Sputum was coughed out following a deep breath, collected into a sterile, screw capped container. If an adequate specimen cannot be obtained, then production was induced with heated aerosol saline suspension
Bronchoscopy	Bronchial brushing, biopsy or broncho alveolar lavage fluid collected and transported in sterile sealed container
Cerebrospinal fluid(CSF)	As much CSF as possible collected aseptically transported in sterile sealed container and processed without delay. If delayed sample left at room temperature
Urine	First early morning clean catch mid stream urine specimen collected aseptically in sterile, screw capped container and processed immediately. If delay of >2hrs anticipated, sample should be refrigerated at $4^{o}C$
Exudates	After using disinfectant, exudates from deep abscess site aspirated using sterile needle and syringe.

Tissue biopsy	Biopsy from lesion site taken and transported in a sterile gauze moistened with physiologic, non bacteriostatic, sterile saline solution in a screw capped container
Blood	After disinfecting the venipuncture site with 70% alcohol, 2 sets of blood sample collected from right and left arm(>20ml/set) at the time of febrile episode in a sterile culture broth

GUIDELINES FOR SPECIMEN COLLECTION:[82]

- Specimen must be collected from an active lesion containing viable organisms
- Specimen must be collected under sterile aseptic precautions
- Sufficient specimen must be collected
- Specimen must be collected before instituting therapy
- Sterile collection devices and containers must be used
- Specimen must be labeled appropriately

CRITERIA FOR REJECTION: [82]

- Unlabelled samples
- Discrepancy between the information on the request form and the container label
- Specimen transported at improper temperature
- Specimen transport time if delayed
- Samples received in unsterile container

- Samples collected in insufficient volume
- Samples that have leaked or show signs of drying
- Samples received in formalin

PROCESSING OF SPECIMENS:[82]

All specimen are microscopically examined and cultured as soon as possible.When the specimen reach the laboratory, they are appropriately processed to ensure viability of the etiological agent and to minimize the chance of contamination.

SPECIMEN	PROCESSING OF SPECIMEN
Respiratory specimen	Most purulent or blood flecked parts of sample are selected to culture. Highly viscid sample was homogenized by adding a pinch of crystalline N-acetyl-L-Cystine. Since respiratory secretions were contaminated with bacteria, media containing antibiotics was used for primary isolation
Cerebrospinal fluid(CSF)	CSF samples centrifuged at 1500-2000g for 20min and inoculated on non inhibitory culture media.
Urine	About 10ml of urine sample centrifuged, 0.5ml of sediment inoculated on both inhibitory and non inhibitory agar medium.
Tissue	Tissue was minced into 1mm cubes with sterile scissors or a sharp scalpel blade and the tiny fragments were placed directly on agar& submerging them slightly beneath 5-10 ml of tissue homogenate, bone marrow or body fluids were sedimented and placed on non selective culture media since the samples were sterile
Blood	5-10 ml of blood sample inoculated in biphasic media containing brain heart infusion broth and agar.

DIRECT EXAMINATION:[82,83]

POTASSIUM HYDROXIDE (KOH) MOUNT PREPARATION:

On a clean, grease free glass slide one large drop of 10% KOH was placed and a small quantity of specimen was mixed with it. A sterile coverslip was placed over the drop. The slide kept in a moist chamber at room temperature for 10 minutes to allow the debris to clear. Observed under low and high power of the light microscope for the presence of yeasts or hyphal forms.

CALCOFLUOR WHITE MOUNT:

Specimens were mixed with the water soluble, colourless dye Calcofluor white, a fluorescent whitener on the slide, which as an affinity for chitin and cellulose of fungal cell wall and favours demonstration of fungal elements more easier when viewed under fluorescent microscope.

INDIA INK OR 10% NIGROSIN MOUNT: [NEGATIVE STAINING]

When *Cryptococcus neoformans* suspected, India ink mount was prepared with a drop of centrifuged sediment and dye on the center of clean grease free slide and mixed well with a loop and a sterile coverslip placed vertically such that one edge just touches the fluid on the slide. keeping the edge in contact with the fluid surface, coverslip dropped gently o the fluid so that no air bubble is trapped inside. Mount examined microscopically for the presence of encapsulated, budding yeast cell.

SPECIAL STAINING TECHNIQUE:

Histologic evaluation of tissues is a quick and easy way to identify fungal organisms, and a strong adjunct to microbiologic culture for diagnosis of fungal

infections. So Simultaneously all the specimens were also processed in Institute of Pathology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai. Haematoxylin and Eosin stain was done routinely. Special stains like Giemsa, Gomori Methenamine silver were also performed when fungal forms suspected in H&E.

ANTIGEN DETECTION BY LATEX AGGLUTINATION TEST (LAT) - The supernatant from the centrifuged CSF sample was inactivated by placing in a boiling water bath for 5 minutes to reduce non-specific interference in the test. The kit used was Cryptococcal Antigen Latex Agglutination System (CALAS), [Meridian Biosciences]. The following procedure was followed-

1. One drop of positive control was added to each of the two designated rings.
2. 25 µl of the antibody control and negative control were added to the appropriate rings.
3. 25 µl of CSF sample was added to each of the two designated rings.
4. One drop of detection latex was added to each of the designated rings.
5. Similarly, one drop of control latex was added to each of the designated rings.
6. The contents of the rings were mixed using separate applicator sticks.
7. The card was rotated at 125 rpm for 5 minutes on a rotator and the reaction was graded as follows:
    a) Negative: homogenous suspension of particles with no visible clumping
    b) 1+ : fine granulation against a milky background
    c) 2+: small but definite clumps against a slightly cloudy background

d) 3+: large and small clumps against a clear background

e) 4+ large clumps against a very clear background

A reaction of 2+ or more with the detection latex was taken as positive for the presence of cryptococcal polysaccharide antigen in the CSF

CULTURE:[82]

A minimum amount of specimen was inoculated onto 2 slants of Sabouraud Dextrose Agar of pH 5.6 with antibiotics gentamycin added at a concentration of 20mg/liter[37] . Inoculated tubes were incubated at 25 and 37^{o}C. Tubes were daily examined for a week for growth. Cultures were incubated for a period of 4-6 weeks before discarding the tubes as sterile and negative. Brain heart infusion biphasic culture bottles with antibiotics was used for the recovery of fungi from blood samples.

INTERPRETATION OF FUNGAL CULTURE:

The following features helps in distinguishing contaminants from pathogen[42]:

- ✓ Isolation of same strain in all culture tubes
- ✓ Repeated isolation of same strain in multiple specimens
- ✓ Immune status of the patient
- ✓ Direct microscopic detection of fungal forms

IDENTIFICATION OF FUNGAL ISOLATES:[42]

All isolates were systematically identified by standard techniques

YEAST:

The biochemical rather than morphological criteria used for identification of Yeasts. Biochemical reaction includes ability to assimilate and ferment sugars, Germ tube production, Chlamydospores production on corn meal agar. Special media like CHROMagar or Caffeic acid agar.

CAFFEIC ACID AGAR:

Pathogenic Cryptococcus neoformans when grown on the medium containing Caffeic acid agar produces dark or black colonies on incubating at 35^{o}C for 3-5 days, because of phenol oxidase produced by organism oxidizes caffeic acid into melanin.

CHROM AGAR SYSTEM:

This is a novel differential and selective culture medium for isolation and presumptive identification of different species of *Candida*. The species of *Candida* can be identified by different coloured colonies produced due to the reaction between specific enzymes of the different species and the chromogenic substrates in the system, which are as follows:

- *C.albicans*-light green
- *C.dubliniensis*- dark green
- *C.glabrata*-pink to purple
- *C.krusei*-pink

- *C.parapsilosis*-cream to pale pink
- *C.tropicalis*-blue with pink halo

GERM TUBE PRODUCTION(REYNOLDS-BRAUDE PHENOMENON)

The culture of *Candida species* was treated with 0.5ml of serum and incubated at 37^{o}C for 2 hours. A drop of suspension was kept on the slide and examined under the microscope. The germ tubes were seen as long tube like projections extending from the yeasts cells.

SUGAR ASSIMILATION:

6.7% Yeast nitrogen base with 2% agar concentration(twin pack Himedia,Mumbai) plates were prepared and carbohydrate containing filter paper discs with 4% concentration were placed and incubated at 35^{o}C overnight. Carbohydrate utilization is determined by growth around disc[42,123].

Fungus	Maltose	Sucrose	Galactose	Cellobiose	Inositol	Raffinose	Dulcitol	Starch
C.albicans	+	+	+	0	0	0	0	+
C.tropicalis	+	+	+	+	0	0	0	+
C.glabrata	0	0	0	0	0	0	+	0
C.parapsilosis	+	+	+	0	0	0	0	0
C.neoformans	+	+	+	+	+	+	0	+

SUGAR FERMENTATION:

1% peptone water supplemented with different carbohydrates of 2% concentration, a colour indicator to assess the pH changes to measure acid production and a tool to assess gas production(by using an inverted Durhams tube). The tubes were incubated at 30^0C for 24-48 hours. The colour change in the tube containing the particular sugar indicates the yeast's ability to ferment carbohydrate[42,123].

Fungus	**Glucose**	**Maltose**	**Sucrose**	**Lactose**	**Galactose**	**Trehalose**
C.albicans	+	+	0	0	+	+
C.tropicalis	+	+	+	0	+	+
C.glabrata	+	0	0	0	0	0
C.parapsilosis	+	0	0	0	0	0

UREASE TEST:

Detection of urease activity of *Cryptococcus* was performed using Christensen's urease medium. Using a loop, a small amount of pure growth from a 48-72hr old culture was inoculated on urease slants and the tubes were incubated at 30°C. If urease is present in the isolate, urea is split to ammonia which raises the pH turning the slant to deep pink colour due to the presence of phenol red indicator. The urease test was declared negative only after 4 days. ATCC *C.neoformans* 32045 and ATCC *C.albicans* 90028 were used as positive and negative controls respectively.

YEAST IDENTIFICATION SCHEME[83]:

This includes,

- Culture characteristics- colony colour, shape and texture
- Asexual structure-
    - Shape and size of cells
    - Type of budding- unipolar, bipolar, multipolar, fission
    - Presence or absence of arthroconida, blastoconidia, germ tubes, hyphae, pseudohyphae,
- Sexual structure- arrangement , cell wall, ornamentation, number, shape and size of ascospores or basidiospores
- Physiological studies-
    - Germ tube test
    - Sugar fermentation
    - Sugar assimilation
    - Urea hydrolysis

FILAMENTOUS FUNGI:

MICROSCOPY: Various mounting methods performed includes:

a. Tease mount

b. Scotch tape

c. Slide culture technique

Tease mount technique:

On a clean grease free microscopic slide a drop of lactophenol cotton blue(LPCB) was placed. A small portion of growth was removed midway between the colony center and edge, placed on the drop of LPCB on slide. The growth was teased using a pair of dissecting needles so as to have a thin spread out. Coverslip placed gently at the edge of the drop of mounting fluid to avoid trapping of air bubbles[42,83] & mount was examined under microscope.

Scotch tape technique:

A drop of mounting fluid placed on the slide. 2cm long tape was taken with one end touched to a forceps and other end to the colony. The tape with the surface containing fungus was laid into the mounting fluid on slide. The tape was detached from the forceps and mount was examined under microscope.

Slide culture technique[83]:

Setup: In a 100mm glass petridish, a filter paper, V- shaped glass rod, a microscopic slide and a coverslip placed and the whole setup was autoclaved at 121^{o}C for 15 minutes.

Procedure: 1cm square agar block was cut aseptically from potato dextrose agar and transferred to the slide in the setup. A very small amount of the colony was transferred to four sides of the agar block and coverslip was placed on the inoculated agar block. 1-1.5 ml of sterile water was added to filter paper to prevent drying of agar block. 5% glycerin was added to sterile water to prevent condensation of moisture on the slide. Slide culture was incubated in the dark at room temperature till good sporulation occurs.

Taking – down the slide culture: A small drop of mounting fluid (LPCB) was placed on a microscopic slide. With forceps, the coverslip was carefully removed, a drop of 95% alcohol was added to coverslip to wet the colony and to prevent trapping of air bubble and placed carefully on the mounting fluid and was examined under microscope.

MOULD IDENTIFICATION SCHEME[83]:

This includes,

- Growth rate
- Colony characteristics – texture, colony(obverse, reverse), pigmentation
- Microscopy
  - (i) Fruiting structures: Synnemata, Pycnidia, Ascocarps
  - (ii) Hyphae : Colour, Size, Septation, Special structures
  - (iii) Conidiogenesis : Conidiogenous cell, Proliferation of conidiophores

ANTIFUNGAL SUSCEPTIBILITY TESTING[42,82-84,124]

Antifungal susceptibility testing of yeast and filamentous fungi was performed as per CLSI guidelines. It was done by Kirby-Bauer disc diffusion method, Epsilometer test, Broth dilution method and agar dilution method.

MIC – MICROBROTH DILUTION METHOD

Amphotericin B powders were obtained from Himedia, Mumbai. Itraconazole,Voriconazole and Fluconazole powders were obtained from Pharma Fabricon, Madurai. Their potency were 750µg/mg each.

STOCK SOLUTION:

For Water soluble drugs like Fluconazole sterile water was used as solvent, whereas for water insoluble drugs like Amphotericin B, Itraconazole and Voriconazole,Dimethyl sulfoxide(DMSO) was used. Stock solution of 5200µg/ml for Fluconazole and 1600µg/ml for other drugs was prepared. A series of dilutions at 100 times the final concentration was prepared from stock solution in RPMI for Fluconazole and with DMSO for others. Each intermediate solution was then further diluted to final strength in RPMI test medium.

$$\text{Weight (mg)} = \frac{\text{Volume(ml) x desired concentration(µg/ml)}}{\text{Assay potency(µg/ml)}}$$

$$\text{Volume (ml)} = \frac{\text{weight(mg) x Assay potency(µg/ml)}}{\text{concentration(µg/ml)}}$$

TEST MEDIUM USED: RPMI 1640(with glutamine, without bicarbonate, Phenol red as pH indicator, Himedia ,Mumbai) with MOPS 0.165M

***INOCULUM PREPARATION*:**

YEAST (CLSI M27-A2 protocol): All Yeast fungi were subcultured on Sabouraud dextrose agar, incubated at 35^{o}C for 24hours for *Candida sp* and 48hours for *C.neoformans.* Colonies were suspended in sterile saline adjusted to 0.5McFarland. The resulting yeast stock suspension of $1x10^6$ to $5x10^6$ cells/ml converted to working suspension by diluting 1:100 dilution followed by 1:20 dilution with RPMI 1640 broth medium, which results in$5x10^2$ to $2.5x10^3$ cells per ml.

INCUBATION: All plates were incubated at 35^{o}C and examined after 46-50 hours for *Candida sp* and upto 72 hours for *C.neoformans.*

FILAMENTOUS FUNGI (CLSI M38-A protocol): All organisms were subcultured onto Potato dextrose agar, incubated at 35^{o}C for 7 days. The culture was covered with 1ml sterile 0.85% saline and a suspension prepared by gently probing the colonies. 1 drop of Tween 20 was added to help in dispersion of conidia. The resulting mixture of conidia and hyphal elements was withdrawn and transferred to a sterile tube and allowed to settle. The uniform suspension was transferred to a screw capped tube and vortexed. The density of conidia suspension were read and adjusted to a optical density of 0.09-0.11 for *Aspergillus spp* and 0.15 – 0.17 for *Rhizopus spp* by Spectrophotometry. These will be diluted 1:50 in test medium, gives a density of approximately $0.4x10^4$ to $5x10^4$ CFU/ml when mixed with antifungal agent.

INCUBATION: All plates were incubated at 35^{o}C and examined after 21-26 hours for *Rhizopus* and after 46-50 hours of incubation of *Aspergillus spp* and other filamentous fungi.

INTERPRETATION:

Minimum inhibitory concentration is the lowest concentration of an antifungal that substantially inhibits growth of the microorganism as detected visually. Each microdilution well was then given a numerical score;

Score 4- No reduction of growth

Score 3- Slight reduction in growth(75% of growth control)

Score 2-Prominent reduction in growth(50% of growth control)

Score 1- slightly hazy

Score 0 -Optically clear or absence of growth

MIC for Amphotericin B – Score 0 and MIC for Azoles – score 2

One growth well and one antifungal control well were also put in parallel. ATCC *A.flavus* 204304, and ATCC *C.krusei* 6258 & ATCC *C.parapsilosis* 90018 were used as quality control strain for filamentous fungi and yeast respectively. The recommended MIC limits of ATCC *A.flavus* were of 0.5 - 4µg/ml for Amphotericin B, 0.2 – 0.5µg/ml for Itraconazole, 0.5-4 µg/ml for Voriconazole. The recommended MIC ranges of ATCC *C.krusei* 6258 were 1-4µg/ml for Amphotericin B, 16-128 µg/ml for Fluconazole,0.25 – 1µg/ml for itraconazole and 0.25 – 1µg/ml voriconazole. The recommended MIC ranges of ATCC *C.parapsilosis* 90018 were 0.5-2µg/ml for Amphotericin B and 0.25-1µg/ml for Fluconazole.

PROCEDURE:

Procedure for Water soluble Antifungal Agents Used in microbroth Dilution Susceptibility Tests

Drug – Starting Concentration (µg/ml)	Stock 5120	2	4 160	8	16	32 20	64	128	256 2.5	511	Remarks
Tube #	TUBE 1	2X	4X	8X	2X	4X	8X	2X	4X	8X	
		TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	TUBE 10	
Source Add DRUG Amount (ml) +	From Stock 1.0	From Tube 1 1.0 +	From Tube 1 1.0 +	From Tube 3 0.5 +	From Tube 3 0.5 +	From Tube 3 0.5 +	From Tube 6 1.0 +	From Tube 6 0.5 +	From Tube 6 0.5 +	From Tube 9 1.0 +	Step 1 Row 1
Add Solvent RPMI (ml)	7.0	1.0	3.0	1.0	1.5	3.5	1.0	1.5	3.5	1.0	
Intermediate Drug Concentration (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25	
Add Drug from Tube Row 1 Above (ml) +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	Step 2 Row 2 5X (1:4)
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
Final Concentration at 1:5 (µg/ml)	128	64	32	16	8	4	2	1	0.5	0.25	(2X)
From Row 2 add Drug to Micro liter plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	

Procedure for Water Insoluble Antifungal Agents Used in microbroth Dilution Susceptibility Tests

Drug – Starting Concentration (µg/ml)	1,600	2	4	8 200	16	32	64 25	128	258	511	Remarks
Tube #	TUBE 1 (Stock) (100 X)	2X TUBE 2	4X TUBE 3	8X TUBE 4	2X TUBE 5	4X TUBE 6	8X TUBE 7	2X TUBE 8	4X TUBE 9	8X TUBE 10	
Source Add DRUG Amount (ml) +	From Tube 1 -	From Tube 1 0.5 +	From Tube 1 0.5 +	From Tube 1 0.5 +	From Tube 4 0.5 +	From Tube 4 0.5 +	From Tube 4 0.5 +	From Tube 7 0.5 +	From Tube 7 0.5 +	From Tube 7 0.5 +	Step 1 Row 1
Add Solvent DMSO (ml)	-	0.5	1.5	3.5	0.5	1.5	3.5	0.5	1.5	3.5	
Intermediate Drug Concentration (µg/ml)	1,600	800	400	200	100	50	25	12.5	6.25	3.13	
Add Drug from Tube Row 1 Above (ml) +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	Step 2 Row 2 (1:50)
RPMI 1640 (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	
Final Concentration at 1:50 (µg/ml)	32	16	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0625	(2X)
From Row 2 add Drug to Micro liter plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313	

DMSO = Dimethy Sulfoxide

CLINICAL SIGNIFICANCE:

YEAST:

AMPHOTERICIN B: MIC above 1µg/ml have been associated with treatment failure and MIC below 1µg/ml with clinical cure

AZOLES: Organism was read as susceptible to fluconazole when MIC<8 µg/ml, Susceptible Dose Dependent 16-32µg/ml and Resistant if >64µg/ml. For other azoles most isolates of MIC<1µg/ml are associated with clinical cure to the drug. Data are not yet available to indicate a correlation between MIC and outcome of treatment with the new azoles.

FILAMENTOUS FUNGI

AMPHOTERICIN B: MIC above 2µg/ml have been associated with treatment failure and MIC below 2µg/ml with clinical cure

AZOLES: Preliminary data indicate high Itraconazole MIC's (MIC>8µg/ml) are associated with clinical resistance to the drug. Data are not yet available to indicate a correlation between MIC and outcome of treatment with the new azoles.

DISC DIFFUSION:

Inoculum transmittance was adjusted according to CLSI M38-A protocol as described above for microbroth dilution method. Suspension was applied to the surface of Mueller Hinton agar with 2% glucose for disc diffusion[85] using swab applicator(CLSI M44-A) .The inoculated plates were dried for 15 minutes. Amphotericin B 10µg, Fluconazole 25µg and Itraconazole 10µg discs were used (Himedia, Mumbai) [86]. Zone diameter were measured in disc diffusion assay to nearest

whole millimeter at the point where there was a prominent reduction of growth after 16-24 hours for Zygomycetes and after 48 and 72 hours for the other species[125].

The organism were interpreted as Susceptible, Susceptible Dose Dependent, Resistant based on the following zone diameter:[85]:

Drugs	Susceptible	Susceptible Dose Dependent	Resistant
Amphotericin B	>15mm	13 – 14mm	<12mm
Fluconazole	<14mm	15-18mm	>19mm
Itraconazole	>17mm	14 – 16mm	<13mm

EPSILOMETER TEST(E-TEST):

Inoculum suspension for E test was prepared similar to disc diffusion method and plated on RPMI agar [85] using swab applicator. Estrip of Amphotericin B, Itraconazole and Fluconazole (Himedia, Mumbai) was applied onto RPMI agar surface [87]. E test was read after 24 hours or when there was sufficient growth to take a reading[87-90]

AGAR DILUTION:

Stock solution and drug dilutions were prepared according to the CLSI M38A guidelines. For performing this technique, 1ml of Yeast nitrogen base was thoroughly mixed with 18ml of 2% molten agar(Himedia,Mumbai) and 1ml of corresponding drug dilution(0.0313 - 16µg/ml) and poured in sterile petri plates. Plates were dried prior to use. The inoculum was adjusted to $1.0x10^6$ cells per ml establishing 90% transmission at 530nm [91]. 10µl(0.01ml) amount was delivered on agar surface. A control plate without antifungal drug was put. Inoculated plates were incubated at 30^oC for 48 hours. The MIC was defined as the lowest concentration which caused greater than 80% inhibition of growth compared with growth on control plate.

RESULTS

RESULTS

A total of 200 patients satisfying inclusion criteria, admitted with signs and symptoms of Invasive Fungal Infections [IFI] were included in the study. Out of 200 patients, 79 patients showed Fungal growth.

Table 1: Age and Sex distribution among study population (n=200)

Age (years)	Risk Factors												Total n (%)
	Renal Transplant (RT)		Diabetes Mellitus (DM)		RT & DM		Chemo Therapy (CT)		CT & DM		HIV		
	M	F	M	F	M	F	M	F	M	F	M	F	
18-20	3	2	2	1	-	-	5	-	-	-	1	2	16 (8%)
21-30	18	11	4	3	1	-	3	2	-	-	1	1	44(22%)
31-40	14	2	11	7	-	-	6	2	-	-	7	3	52(26%)
41-50	7	1	12	8	3	-	9	-	1	-	3	-	44(22%)
51-60	1	-	11	2	1	-	6	2	-	1	2	2	28(14%)
61-70	-	-	4	2	-	-	7	-	-	-	1	-	14(7%)
71-80	-	-	-	-	1	-	1	-	-	-	-	-	2(1%)
Total n (%)	**43(22)**	**16(8)**	**44(22)**	**23(12)**	**6(3)**	-	**37(19)**	**6(3)**	**1(1)**	**1(1)**	**15(8)**	**8(4)**	**200(100%)**

The above table shows, the study consists of predominantly males (73%) when compared to females (27%).Out of 200 patients majority of them (26%) in the study belong to the age group of 31- 40 years, followed by equal number of distribution among 21-30 and 41-50 years age group (22%).

CHART 1(A): SEX DISTRIBUTION AMONG STUDY POPULATION

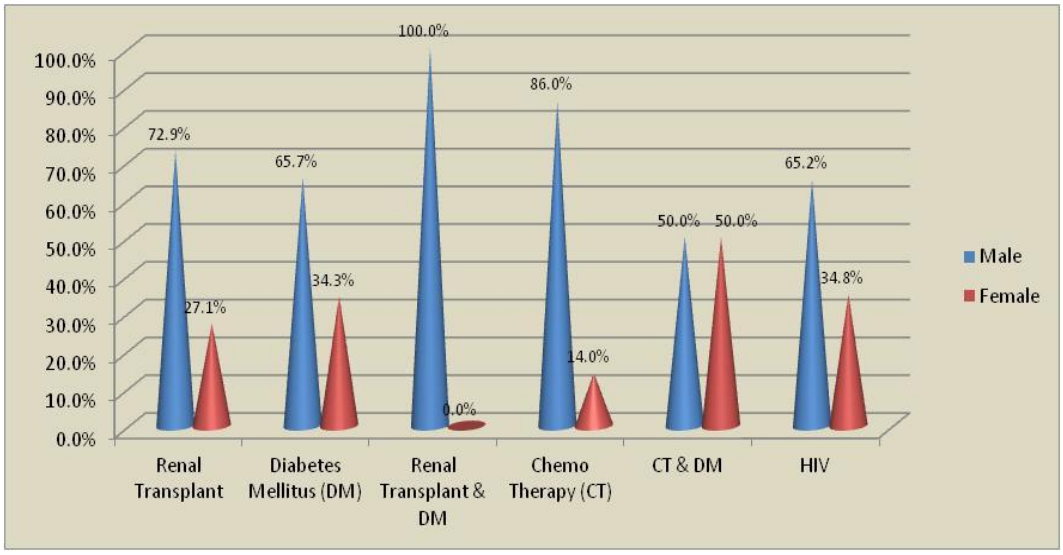


CHART 1(B): AGE DISTRIBUTION AMONG STUDY POPULATION

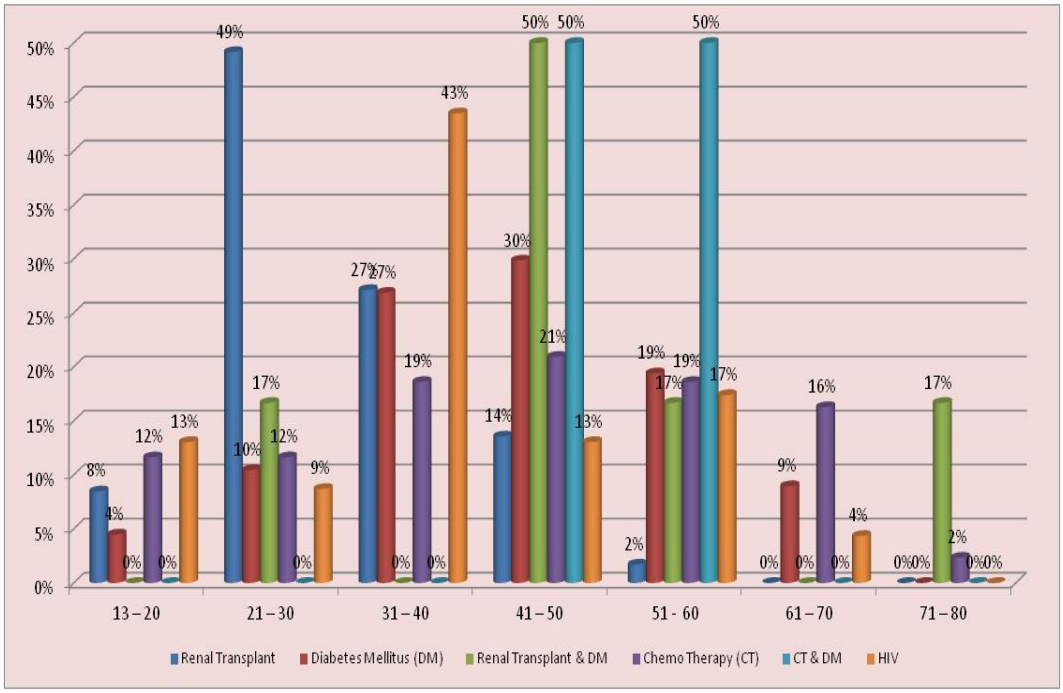


Table 2: Period of Immunosuppression favouring fungal infection (n=200)

Period of Immunosuppression	Criteria						
	Renal Transplant	**Diabetes Mellitus (DM)**	**Renal Transplant & DM**	**Chemo Therapy (CT)**	**CT & DM**	**HIV**	**Total**
< 1 year	27	-	1	-	-	-	28(14%)
1 – 10 years	31	61	5	34	2	21	154(77%)
>10 years	1	6	-	9	-	2	18(9%)
Total	**59(29.5%)**	**67(33.5%)**	**6(3%)**	**43(21.5%)**	**2(1%)**	**23 (11.5%)**	**200**

In this study, Immunosuppressive period of 1 – 10 years showed increased incidence of IFI among 154(77%) patients.

Table 3: Distribution of samples collected from study population with various risk factors (n=200)

Specimen	Risk factors						Total n(%)
	Renal Transplant	Diabetes Mellitus (DM)	Renal Transplant & DM	Chemo Therapy (CT)	CT & DM	HIV	
Urine	23	35	1	-	-	-	59(29.5%)
Sputum	1	3	-	21	-	-	25(12.5%)
Br. Wash	1	15	-	8	1	-	25(12.5%)
Drain / Catheter	20	-	1	-	-	-	23(11.5%)
CSF	1	-	-	-	-	18	19(9.5%)
Pus	4	3	1	1	-	1	10(5%)
Pleural fl	-	2	-	6	-	-	8(4%)
Blood	2	2	2	1	-	-	7(3.5%)
Gastric lavage	2	-	-	2	-	2	6(3%)
BAL	1	3	-	1	-	-	5(2.5%)
Ascitic fl.	1	-	-	3	1	-	5(2.5%)
FNAC aspirate	-	4	1	-	-	-	5(2.5%)
Palatal Scraping	-	-	-	-	-	2	2(1%)
IV Catheter tip	1	-	-	-	-	-	1(0.5%)
Total	59	67	6	43	2	23	200

Among immunosuppressive patients in the study, the majority of samples were Urine (29.5%), Sputum(12.5%) and bronchial wash (12.5%). In HIV positive patients, most predominant sample was CSF (9.5%).

CHART 2: PERIOD OF IMMUNOSUPPRESSION FAVOURING FUNGAL INFECTION

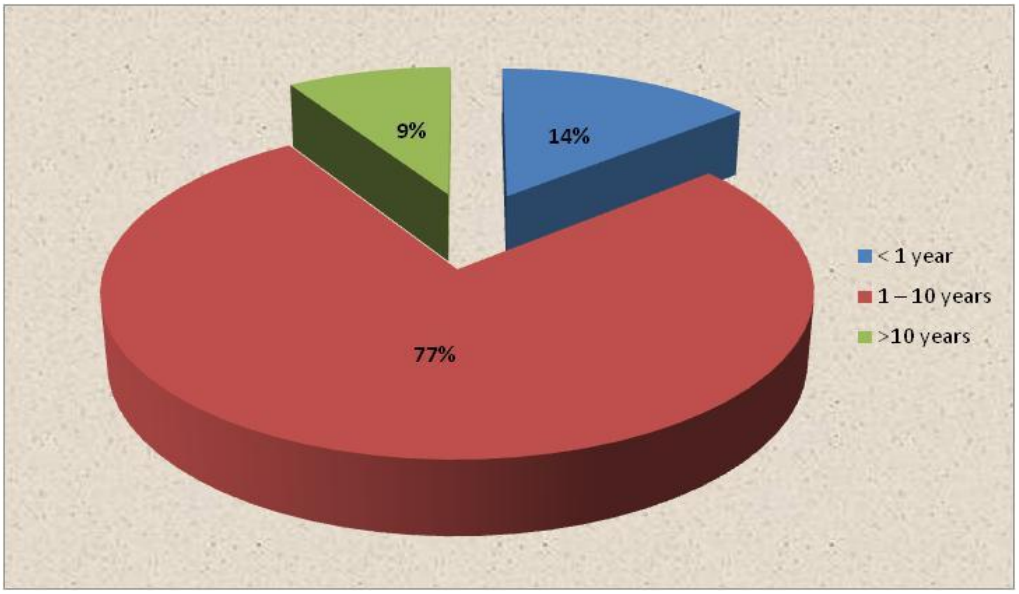


CHART 3: DISTRIBUTION OF SAMPLES COLLECTED FROM STUDY POPULATION

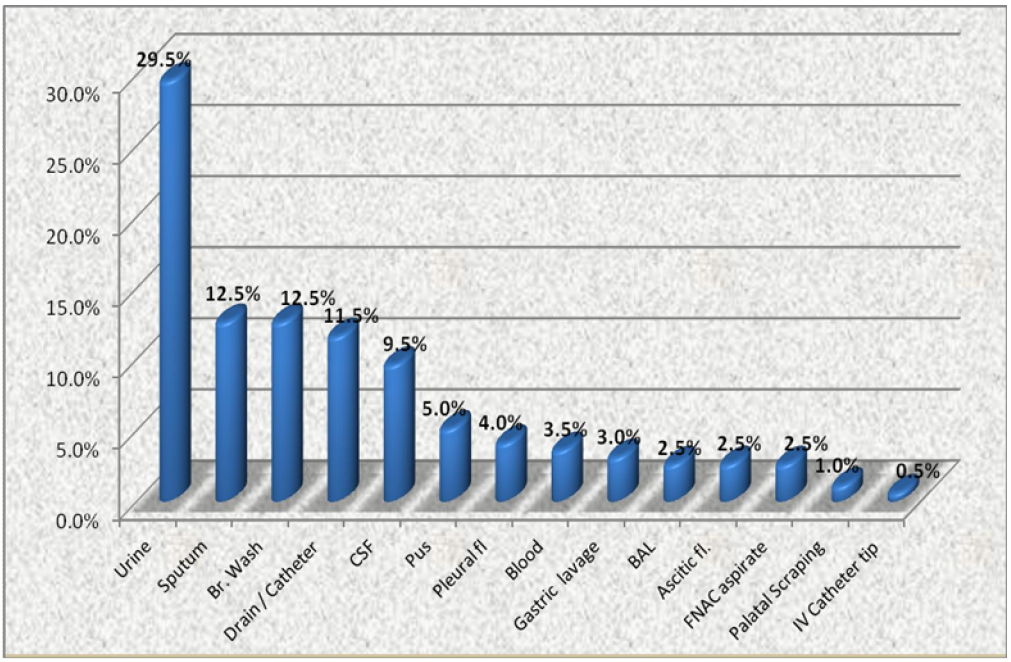


Table 4: **Distribution and Categorization of IFI among suspected study population**(n=200)

Categories	Criteria						Total	
	Renal Transplant	Diabetes Mellitus (DM)	Renal Transplant & DM	Chemo Therapy (CT)	CT & DM	HIV	n	%
Proven	3(5%)	14(21%)	3(50%)	8(19%)	2(100)	5(22%)	35	17.5
Probable	9(15%)	27(40%)	1(17%)	7(16%)	-	-	44	22
Possible	47(80%)	26(39%)	2(33%)	28(65%)	-	18(78%)	121	60.5
Total (%)	59(29.5)	67(33.5)	6(3)	43(21.5)	2(1)	23(11.5)	200	100

The above table shows that the incidence of proven IFI was only 17.5%. Most of the cases were under the category of possible IFI (60.5%).

Table 5: Predisposing factors for IFI among Renal Transplant recipients (n=65)

Predisposing factors	Proven IFI	Probable IFI	Possible IFI	Total n(%)
Hepatitis C virus (HCV)	**2(11.7%)**	1(5.88%)	14(82.3%)	17(26.1%)
Cytomegalovirus(CMV)	-	2(16.6%)	10(83.3%)	12(18.4%)
Diabetes mellitus	1(25%)	1(25%)	2(50%)	4(6.15%)
Post Tx maintenance hemodialysis	-	2(20%)	8(80%)	10(15.3%)
Neutropenia	1(7.69%)	2(15.3%)	10(76.9%)	13(20%)
Iron therapy& DM	**2(100%)**	0	0	2(3.07%)
Herpes simplex virus (HSV)	-	2(28.5%)	5(7.42%)	7(10.76%)
Total	6(9.2%)	10(15.3%)	49(75.3%)	65(100%)

Among 65 post renal transplant patients, 6(9.2%) were proven IFI, 10(15.3%) were probable IFI and 49(17.3%) were possible IFI. Diabetes mellitus patients with iron therapy and HCV positivity appears to be the commonest risk factor for proven IFI.

Table 6: Distribution of criteria for Proven IFI (n=35)

Criteria	KOH / HPE +ve	Fungemia	Culture of Sterile Site	India Ink/ latex agglutination test	Total n=35
Renal T_x	3(8.5%)	-	-	-	3(8.5%)
DM	11(31.4%)	2(5.7%)	1(2.8%)	-	14(40%)
Renal T_x & DM	2(5.7%)	1(2.8%)	-	-	3(8.5%)
CT	7(20%)	1(2.8%)	-	-	8(22.8%)
CT & DM	2(5.7%)	-	-	-	2(5.7%)
HIV	1(2.8%)	-	-	4(11.4%)	5(14.2%)
Total	**26(74.2%)**	4(11.4%)	1(2.8%)	4(11.4%)	35(100%)

Among the 4 accepted methods of approval of IFI, in our study most of the cases showed positivity by KOH/HPE i.e 26(74.2%).

Table 7: Distribution of invasive mycoses among proven and probable cases of IFI (n=79)

Incidence of Specific Mycoses	No.	%
Aspergillosis	33	**16.5%**
Candidiasis	30	15%
Hyalohyphomycosis	5	2.5%
Zygomycosis	4	2%
Cryptococcosis	4	2%
Phaeohyphomycosis	3	1%

Of 79 patients with proven & probable IFI, Aspergillosis n=33(16.5%) was most commonly encountered.

CHART 4: DISTRIBUTION AND CATEGORIZATION OF IFI

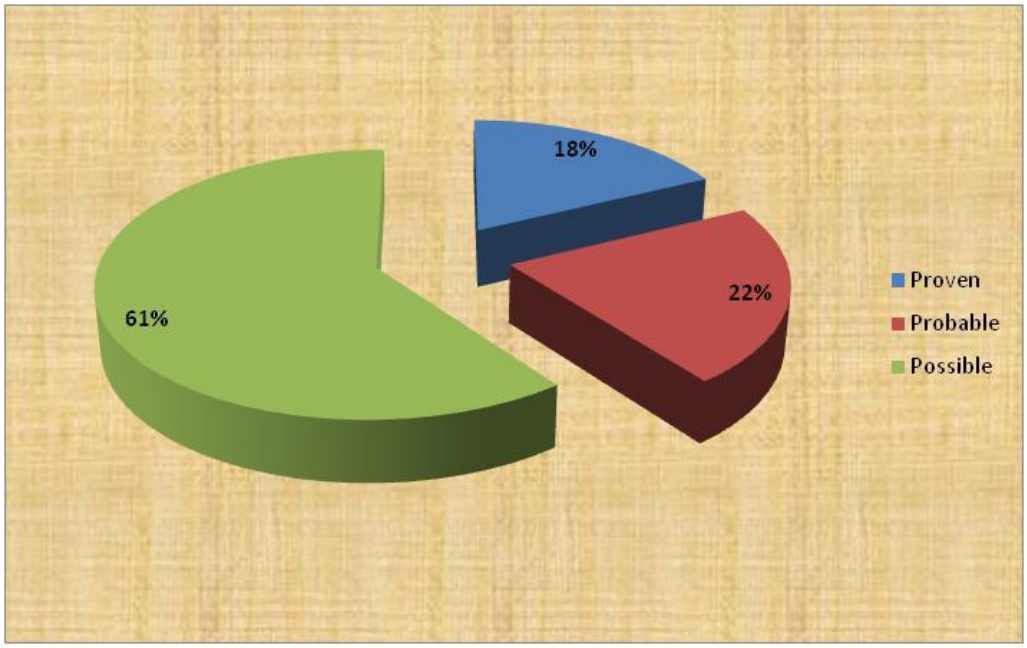


CHART 6: DISTRIBUTION OF CRITERIA FOR PROVEN IFI

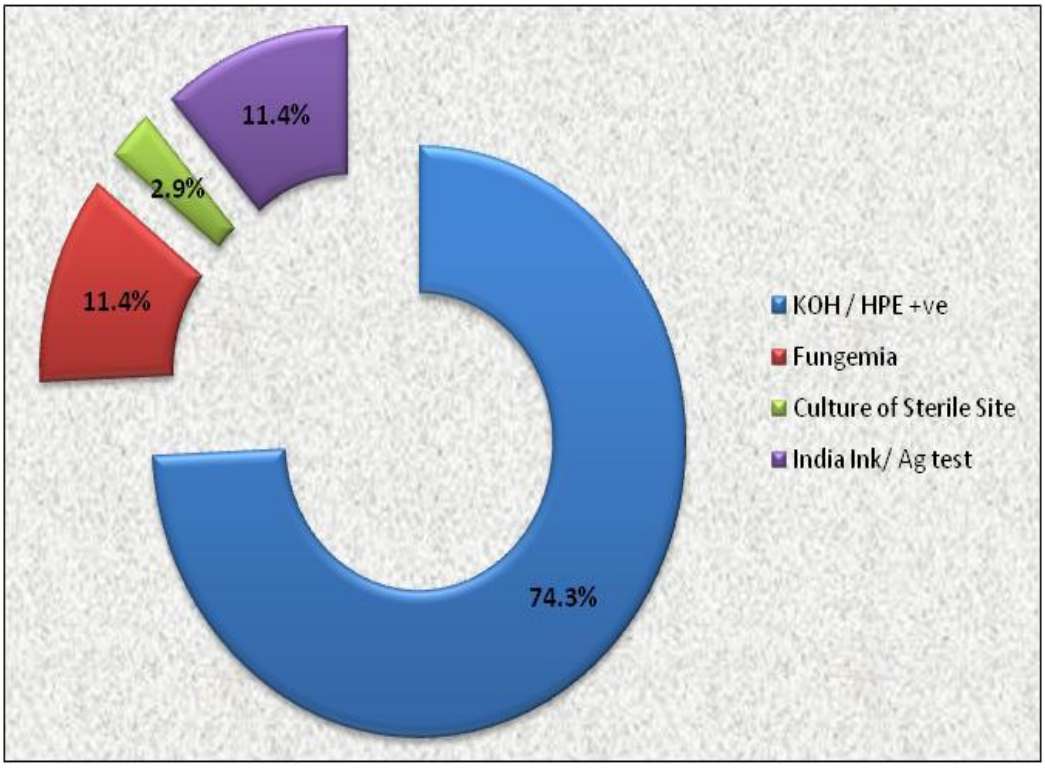


Table 8: Distribution of yeast and yeast like isolates among proven & probable IFI & their frequency of isolation (n=34)

Species	Renal Transplant	Diabetes Mellitus (DM)	Renal Transplant & DM	Chemo Therapy (CT)	CT & DM	HIV	Total n =34
Cryptococcus neoformans	-	-	-	-	-	**4 (80%)**	4 (11.7%)
Candida albicans	5 (55.5%)	9 (64%)	-	4 (100%)	-	1 (20%)	**19 (55.8%)**
Candida tropicalis	2 (22.5%)	2 (14.5)	1 (50%)	-	-	-	5 (14.7%)
Candida glabrata	1 (11%)	2 14.5%)	1 (50%)	-	-	-	4 (11.7%)
Candida parapsilosis	1 (11%)	1 (7%)	-	-	-	-	2 (5.8%)
Total	9(26.4%)	14(41.1%)	2(5.8%)	4(11.7%)	0	5(14.7%)	34

Among the Yeast (34) isolates *Candida spp* 30 (88.2%) was common among study group. Most common species isolated was *C.albicans* 19 (55.8%), followed by 11 (32.3%) *Non Candida albicans*. Most common among NCA was *C.tropicalis* 5 (14.7%) followed by *C.glabrata* 4 (11.7%) and *C.parapsilosis* 2(5.8%). Rest of the 4 (11.7%) were *Cryptococcus neoformans* , which was isolated only from HIV positive patients.

CHART 7: DISTRIBUTION OF INVASIVE MYCOSES AMONG PROVEN AND PROBABLE CASES OF IFI

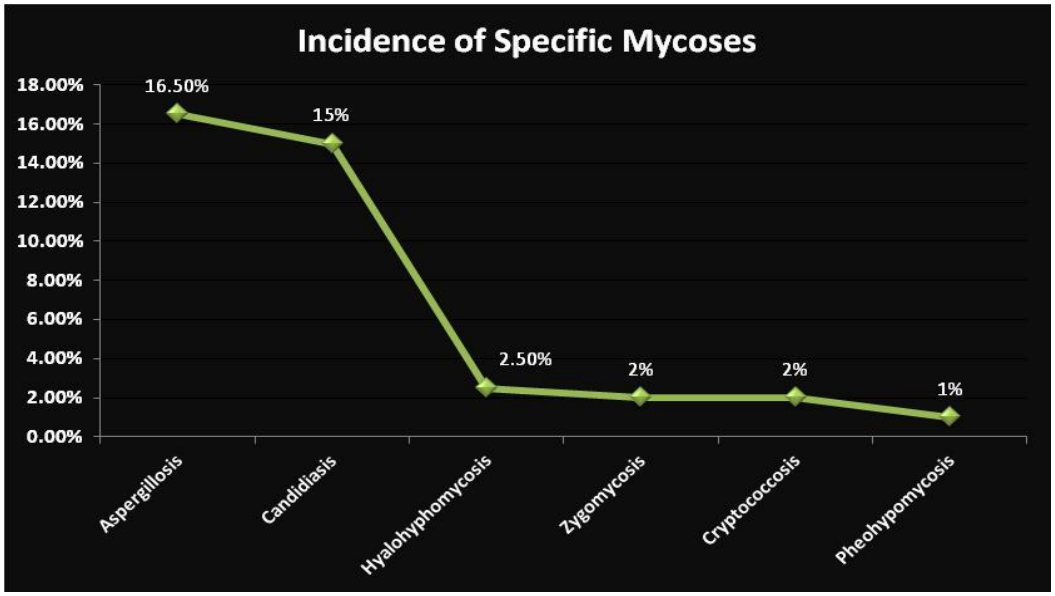


CHART 8: DISTRIBUTION OF YEAST AND YEAST LIKE ISOLATES AMONG PROVEN & PROBABLE IFI

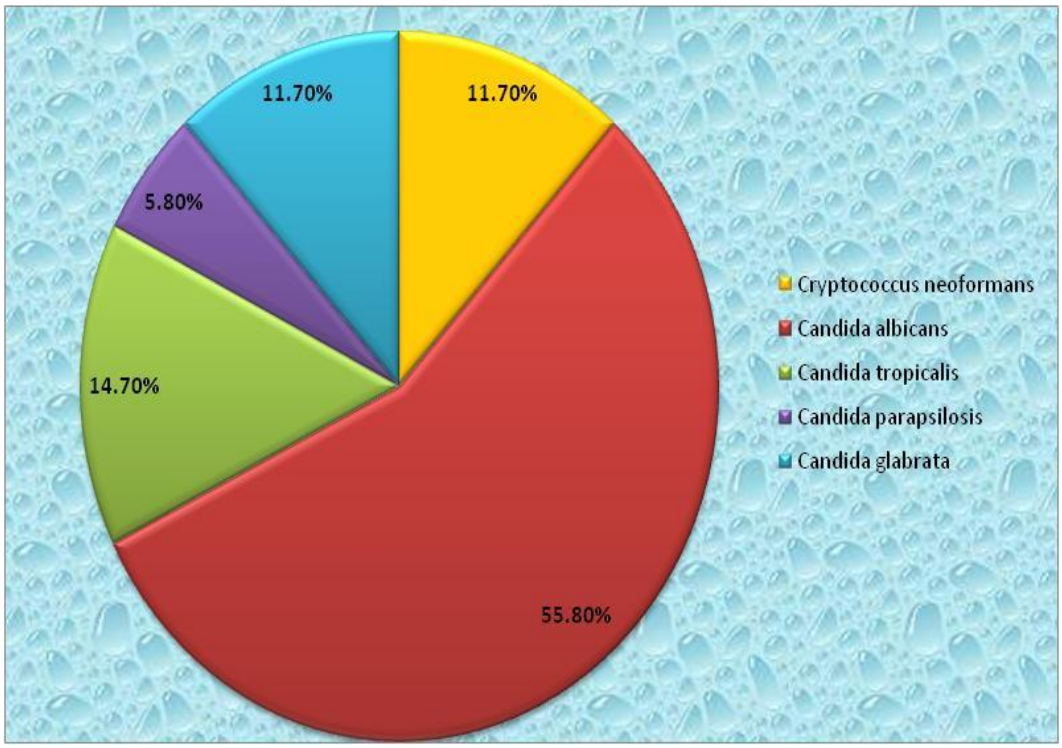


Table 9: Distribution of Filamentous fungal isolates of IFI & their frequency of isolation (n=45)

Species	Renal Transplant	Diabetes Mellitus (DM)	Renal Transplant & DM	Chemo Therapy (CT)	CT & DM	HIV	Total n =45
Asp.fumigatus	2 (66.6%)	**12 (44.4%)**	-	5 (45.5%)	-	-	**19 (42.2%)**
Asp.flavus	-	3 (11.1%)	-	3 (27.2%)	2 (100%)	-	8 (17.7%)
Asp.terreus	-	4 (14.8%)	-	-	-	-	4 (8.8%)
Asp.niger	-	1 (3.7%)	-	-	-	-	1 (2.2%)
Asp.nidulans	-	1 (3.7%)	-	-	-	-	1 (2.2%)
Penicillium spp	-	2 (7.4%)	-	1 (9%)	-	-	3 (6.6%)
Fusarium solani	-	-	-	2 (18.1)	-	-	2 (4.4%)
Rhizopus oryzae	-	1 (3.7%)	2 (100%)	-	-	-	3 (6.6%)
Absidia corymbifera	-	1 (3.7%)	-	-	-	-	1 (2.2%)
Curvularia lunata	-	1 (3.7%)	-	-	-	-	1 (2.2%)
Exserohilum rostratum	-	1 (3.7%)	-	-	-	-	1 (2.2%)
Dematiaceous fungi	1 (33.3%)						1 (2.2%)
Total	3(6.5%)	**27(58.6%)**	2(4.3%)	11(23.9%)	2(4.3%)	-	45

Among the 45 Filamentous isolates, majority of fungi isolated were *Aspergillus spp* 33 (73%), in particular *Aspergillus fumigatus* n=19 (42.2%). And most of them were from patients with uncontrolled DM i.e, 27(58.6%). One dematiaceous fungi isolated from renal transplant recipient was not able to be identified.

CHART 9: DISTRIBUTION OF FILAMENTOUS FUNGAL ISOLATES OF IFI

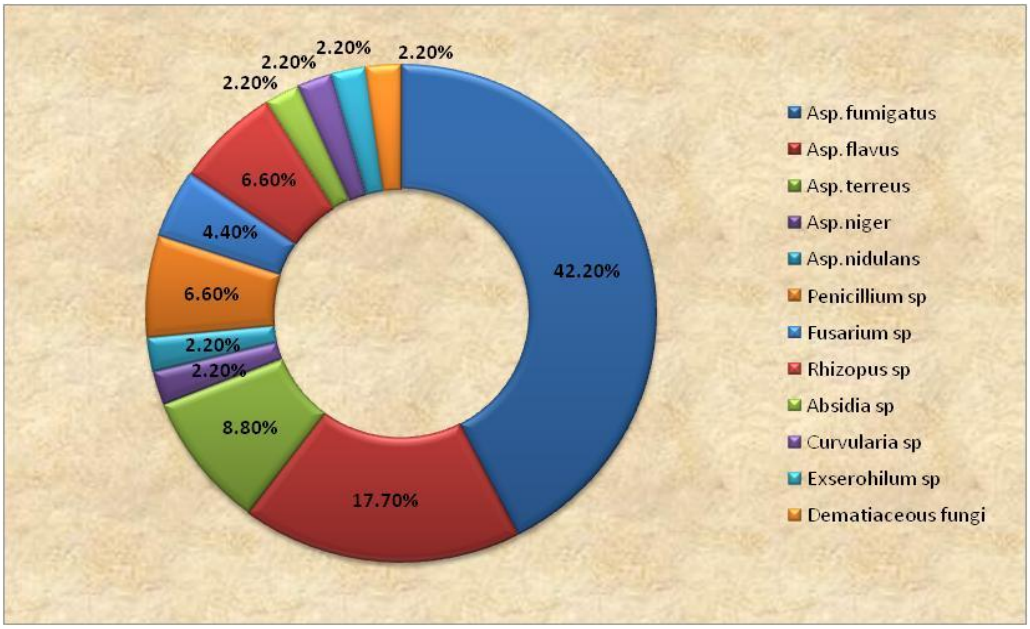


CHART 10: INTERPRETATION OF FOLLOW UP OF THE STUDY CASES:

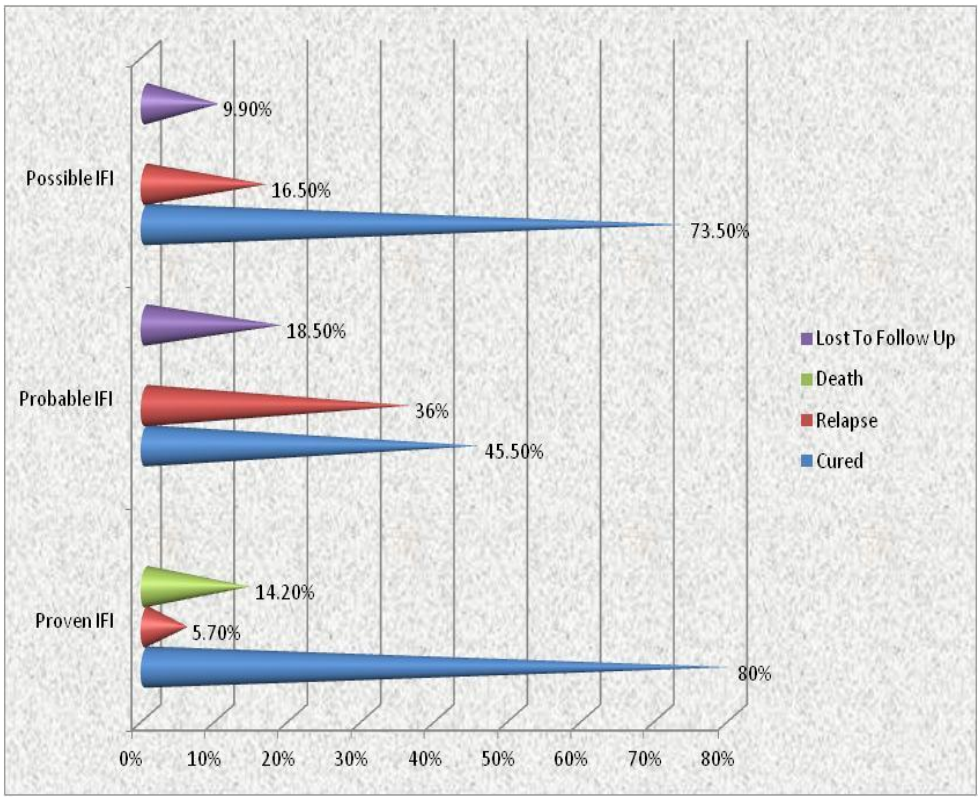


Table 10: Distribution of yeast isolates causing IFI in various samples

Specimen	***C.albicans***	***C.tropicalis***	***C.gladrata***	***C.parapsilosis***	***Cryptococcus neoformans***	**No growth**
Urine(59)	9(15.2%)	5(8.4%)	2(3.2%)	2(3.2%)	-	41(69%)
Drain / Catheter(23)	1(4%)	-	-	-	-	22(96%)
CSF (19)	-	-	-	-	4(21%)	15(79%)
Pus(10)	2(20%)	-	-	-	-	-
Blood (7)	3(43%)	-	2(28.5%)	-	-	2(28.5%)
Gastric lavage(6)	3(66%)	-	-	-	-	3(66%)
IV Catheter tip(1)	1(100%)	-	-	-	-	-

Above table shows that *C.albicans*(15.2%) was commonly reported followed by *C.tropicalis*(8.4%), *C.glabrata*(3.2%) and *C.parapsilosis*(3.2%) from urine specimen. In blood, *C.albicans* 43% were reported followed by 28.5% of *C.glabrata*.

Table 11: Distribution of filamentous fungi causing IFI in various samples:

Specimen	*A.fumigatus*	*A.flavus*	*A.terreus*	*A.niger*	*A.nidulans*	*Penicillium*	*F.solani*	*Rhizopus oryzae*	*Absisia cormbifera*	*Curvularia lunata*	*Exserohilm rostratum*	Unidentified fungi	No growth
Sputum (25)	5(20%)	1(4%)	-	1(4%)	-	-	2(8%)	-	-	-	1(4%)	-	15(60%)
Br. Wash (25)	11(44%)	4(16%)	1(4%)	-	-	3(12%)	-	-	-	1(4%)	-	-	5(20%)
Pus(10)	-	1(10%)	1(10%)	-	-	-	-	1(10%)	-	-	-	1(10%)	4(40%)
Pleural fl (8)	1(12.5%)	-	1(12.5%)	-	-	-	-	-	-	-	-	-	6(75%)
BAL (5)	2(40%)	-	1(20%)	-	1(20%)	-	-	-	-	-	-	-	1(20%)
Ascitic fl. (5)	-	1(20%)	-	-	-	-	-	-	-	-	-	-	4(80%)
FNAC aspirate(5)	-	1(20%)	-	-	-	-	-	2(40%)	1(20%)		-	-	1(20%)
Palatal Scraping(2)	-	-	-	-	-	-	-	-	-	-	-	-	2(100%)

Above table shows, that *A.fumigatus* was most commonly isolated from bronchial wash and sputum. *Rhizopus oryzae* was mostly isolated from orbital bone lesions obtained by FNAC aspiration.

Table 12: Results of minimum inhibitory concentration (MIC) by microbroth dilution method of Amphotericin B, Itraconazole,Voriconazole and Fluconazole to yeast and yeast like fungus

Species	Number	AMPHOTERICIN B			ITRACONAZOLE			VORICONAZOLE			FLUCONAZOLE		
		S* MIC ≤1µg / ml	R MIC >1µg/ml	Mean MIC of Sensitive Isolates (µg/ml)	S* MIC ≤1µg / ml	R MIC >1µg/ml	Mean MIC of Sensitive Isolates (µg/ml)	S* MIC ≤1µg / ml	R MIC >1µg/ml	Mean MIC of Sensitive Isolates (µg/ml)	S* MIC ≤8µg / ml	R MIC >64µg/ml	Mean MIC of Sensitive Isolates (µg/ml)
Cryptococcus neoformans	4	4 (100%)	-	0.0625	-	-	-	4 (100%)	-	0.125	4 (100%)	-	0.125
Candida albicans	19	18 (94.8%)	1 (5.2%)	0.5	18 (94.8%)	1 (5.2%)	0.5	19 (100%)	-	0.25	18 (94.8%)	1 (5.2%)	0.5
Candida tropicalis	5	5 (100%)	-	0.25	5 (100%)	-	0.25	5 (100%)	-	0.125	5 (100%)	-	0.25
Candida parapsilosis	2	2 (100%)	-	0.125	2 (100%)	-	0.0625	2 (100%)	-	0.125	2 (100%)	-	0.0625
Candida glabrata	4	4 (100%)	-	0.25	4 (100%)	-	0.5	4 (100%)	-	0.5	-	4 (100%)	-

S= Sensitive, R=Resistant

*Interpretive criteria currently not standardized. Studies show that for Yeast fungi MIC below 1µg/ml to Amphotericin B, Itraconazole & Voriconazole and MIC below 8µg/ml to Fluconazole are associated with clinical cure. Most of the Yeast isolates in the study were sensitive to all antifungal drugs tested and their MIC were in the range of 0.0313 to 0.125 µg/ml for Amphotericin B, 0.0625 to 0.5 µg/ml for Itraconazole, 0.0625 to 0.5 µg/ml for Voriconazole and 0.0313 to 0.5 µg/ml for Fluconazole.

One *C.albicans* isolate has showed MIC in the resistance range for Amphotericin B, Itraconazole and Fluconazole, but sensitive to Voriconazole. 100% resistance to Fluconazole was seen among *C.glabrata*.

Table 13: Results of MIC by Agar dilution method of Amphotericin B, Itraconazole,Voriconazole and Fluconazole to yeast and yeast like fungus

Species	Number	AMPHOTERICIN B			ITRACONAZOLE			VORICONAZOLE			FLUCONAZOLE		
		S*	R	Mean MIC of Sensitive Isolates (µg/ml)	S*	R	Mean MIC of Sensitive Isolates (µg/ml)	S*	R	Mean MIC of Sensitive Isolates (µg/ml)	S*	R	Mean MIC of Sensitive Isolates (µg/ml)
Cryptococcus neoformans	4	4 (100%)	-	0.0625	-	-	-	1 (100%)	-	0.125	4 (100%)	-	0.0625
Candida albicans	19	18 (94.8%)	1 (5.2%)	0.125	19 (100%)	-	0.125	19 (100%)	-	0.125	19 (100%)	-	0.125
Candida tropicalis	5	5 (100%)	-	0.5	5 (100%)	-	0.25	5 (100%)	-	0.25	5 (100%)	-	-
Candida parapsilosis	2	2 (100%)	-	0.125	2 (100%)	-	0.0625	2 (100%)	-	0.0313	2 (100%)	-	0.125
Candida glabrata	4	4 (100%)	-	0.125	4 (100%)	-	0.125	4 (100%)	-	0.125	-	4 (100%)	0.125

Most isolates were sensitive to Amphotericin B, Itraconazole and Voriconazole by Agar dilution method of detecting MIC. 5.2% of *C.albicans* were resistance to Amphotericin B and 100% resistance to Fluconazole was seen among *C.glabrata*.

Table 14: Results of MIC by E test method to yeast and yeast like fungus to Amphotericin B, Itraconazole and Fluconazole:

Species	Number	AMPHOTERICIN B			ITRACONAZOLE			FLUCONAZOLE		
		S	R	Mean MIC of Sensitive Isolates (µg/ml)	S	R	Mean MIC of Sensitive Isolates (µg/ml)	S	R	Mean MIC of Sensitive Isolates (µg/ml)
Cryptococcus neoformans	4	4 (100%)	-	0.0625	-	-	-	4 (100%)	-	0.0625
Candida albicans	19	18 (94.8%)	1 (5.2%)	0.125	19	-	0.125	19 (100%)	-	0.125
Candida tropicalis	5	5 (100%)	-	0.5	5 (100%)	-	0.25	5 (100%)	-	0.125
Candida parapsilosis	2	2 (100%)	-	0.125	2 (100%)	-	0.0625	2 (100%)	-	0.0625
Candida glabrata	4	4 (100%)	-	0.125	4 (100%)	-	0.125	-	4 (100%)	-

Most of the isolates were sensitive to Amphotericin B and Fluconazole by Epsilometer test and mean MIC range was from 0.0313 to 0.5µg/ml. 94.8% of Candida albicans were sensitive to Amphotericin B and 5.2% were resistant. 100% resistance was seen for Fluconazole among C.glabrata. Universally 100% sensitivity was seen with Itraconazole among all isolates.

Table 15: Results of Disc diffusion test for Yeast and Yeast like fungi for Amphotericin B, Itraconazole and Fluconazole:

Species	Number	AMPHOTERICIN B			ITRACONAZOLE			FLUCONAZOLE		
		S (Zone ≥15mm)	SDD/I(Zone 13-14mm)	R(Zone ≤12mm)	S (Zone ≥17mm)	SDD/I(Zone 14-16mm)	R(Zone ≤13mm)	S (Zone ≥19mm)	SDD/I(Zone 15-18mm)	R(Zone ≤19mm)
Candida albicans	19	15 (79%)	2 (10.4%)	2 (10.4%)	17 (89.6%)	1 (5.2%)	1 (5.2%)	14 (73.6%)	3 (15.7%)	2 (10.4%)
Candida tropicalis	5	5 (100%)	-	-	5 (100%)	-	-	4 (80%)	-	1 (20%)
Candida parapsilosis	2	2 (100%)	-	-	2 (100%)	-	-	2 (100%)	-	-
Candida glabrata	4	4 (100%)	-	-	4 (100%)	-	-	-	-	4 (100%)

S= SUSCEPTIBLE; SDD= SUSCEPTIBLE DOSE DEPENDENT; R= RESISTANT

79% of *C.albicans* were sensitive to Amphotericin B, 10.4% were resistant and 10.4% were susceptible dose dependent. All other isolates were sensitive to Amphotericin B.

89.6% of *C.albicans* were sensitive to Itraconazole, 5.2% were susceptible dose dependent and 5.2% were resistant. All other isolates were sensitive to Itraconazole.

Disc diffusion test using Fluconazole showed that73.6% of *C.albicans* were sensitive, 15.7% were susceptible dose dependent and 10.4% were resistant. Among *C. tropicalis* 80% were sensitive and 20% were resistant. *C.glabrata* showed 100% resistance. All *C.parapsilosis* were 100% sensitive.

Table 16: Comparison of Disk diffusion, E Test and Agar dilution of Amphotericin B with Microdilution Reference Method to Yeast and Yeast like Organisms:

Species	Broth dilution(S)	Disk diffusion			E test		Agar Dilution	
		m	M	VM	M	VM	M	VM
Cryptococcus neoformans(4)	4	-	-	-	-	-	-	-
Candida albicans (19)	18	**2(10.4%)**	**1(5.2%)**	-	-	-	-	-
Candida tropicalis (5)	5	-	-	-	-	-	-	-
Candida glabrata (4)	4	-	-	-	-	-	-	-
Candida parapsilosis(2)	2	-	-	-	-	-	-	-

m = minor error; M= Major error; VM= Very Major error

Interpretated as per the criteria given by A.Espinel –Ingroff et al:

Minor error(m): Shifts between susceptible and susceptible dose dependent or between resistant and susceptible dose dependent

Major error(M): Isolate resistant by other methods but susceptible by broth dilution

Very Major error(VM): Broth dilution shows resistance and others show as sensitive

There is an 100% agreement between microbroth dilution, agar dilution and E test for Amphotericin B. But minor error of 10.4% and a major error of 5.2% was seen between broth dilution and disc diffusion test.

Table 17: Comparison of Disk diffusion, E Test and Agar dilution of Itraconazole with Microdilution Reference Method to Yeast and Yeast like Organisms:

Species	Broth dilution(S)	Disk diffusion			E test		Agar Dilution	
		m	M	VM	M	VM	M	VM
Candida albicans (19)	18	**1(5.2%)**	-	-	-	-	-	**1(5.2%)**
Candida tropicalis(5)	5	-	-	-	-	-	-	-
Candida glabrata (4)	4	-	-	-	-	-	-	-
Candida parapsilosis(2)	2	-	-	-	-	-	-	-

There is an 100% agreement seen between broth dilution and Etest for itraconazole. But a very major error of 5.2% with *C.albicans* was seen between broth and agar dilution and 5.2% of minor error seen between broth and agar dilution.

Table 18:Comparison of Agar dilution of voriconazole with Microdilution Reference Method to Yeast and Yeast like Organisms:

Species	Broth dilution(S)	Agar Dilution	
		M	VM
Cryptococcus neoformans(4)	4	-	-
Candida albicans(19)	19	-	-
Candida tropicalis(5)	5	-	-
Candida glabrata(4)	4	-	-
Candida parapsilosis(2)	2	-	-

100% agreement was seen between broth dilution and agar diffusion for voriconazole.

Table 19: Comparison of Disk diffusion, E test and Agar dilution of Fluconazole with Microdilution Reference Method to Yeast and Yeast like Organisms:

Species	Broth dilution(S)	Disk diffusion			E test		Agar Dilution	
		m	M	VM	M	VM	M	VM
Cryptococcus neoformans(4)	4	-	-	-	-	-	-	-
Candida albicans(19)	18	**3(10.4%)**	**1(5.2%)**	-	-	1**(5.2%)**	-	1**(5.2%)**
Candida tropicalis(5)	5	-	-	-	-	-	-	-
Candida glabrata(4)	4	-	-	-	-	-	-	-
Candida parapsilosis(2)	2	-	-	-	-	-	-	-

On comparing broth dilution with disc diffusion showed a minor error of 10.4% and a major error of 5.2%. On comparing agar dilution and E test with microbroth dilution method showed a very major error of 5.2%.

Table 20: Results of minimum inhibitory concentration(MIC) by microbroth dilution method of Amphotericin B, Itraconazole, Voriconazole and Fluconazole to filamentous fungus

Species	Number	AMPHOTERICIN B			Itraconazole			Voriconazole		
		S* MIC ≤2 µg / ml	R MIC >2 µg/ml	Mean MIC of Sensitive Isolates (µg/ml)	S* MIC ≤8 µg / ml	R MIC >8 µg/ml	Mean MIC of Sensitive Isolates (µg/ml)	S* MIC ≤8 µg / ml	R MIC >8 µg/ml	Mean MIC of Sensitive Isolates (µg/ml)
Asp.fumigatus	19	18 (94.8%)	1 (5.2%)	0.25	17 (89.4%)	2 (10.4%)	0.0313	19 (100%)	-	0.25
Asp.flavus	8	8 (100%)	-	0.5	8 (100%)	-	0.125	8 (100%)	-	0.5
Asp.terreus	4	-	4 (100%)	-	4 (100%)	-	0.5	4 (100%)	-	0.5
Asp.niger	1	1 (100%)	-	0.125	1 (100%)	-	0.125	1 (100%)	-	0.125
Asp.nidulans	1	1 (100%)	-	1	1 (100%)	-	0.5	1 (100%)	-	0.5
Penicillium spp	3	3 (100%)	-	0.5	3 (100%)	-	0.125	3 (100%)	-	0.125
Fusarium solani	2	-	2 (100%)	-	-	2 (100%)	-	2 (100%)	-	0.5
Rhizopus oryzae	3	3 (100%)	-	0.5	3 (100%)	-	0.25	3 (100%)	-	0.5
Absidia corymbifera	1	1 (100%)	-	0.5	1 (100%)	-	0.25	1 (100%)	-	0.125
Curvularia lunata	1	1 (100%)	-	0.5	1 (100%)	-	0.5	1 (100%)	-	0.5

*Interpretive criteria currently not standardized. Studies show MIC of Amphotericin B below 2µg/ml and MIC of Itraconazole and Voriconazole below 8µg/ml to filamentous fungi associated with clinical cure.

Most of the isolates in the study were sensitive to Amphotericin B and were in the MIC range of 0.125 to 1µg/ml for Filamentous forms. 5.2% of *Asp.fumigatus* isolate has shown MIC in resistant range. All *Asp.terreus* and *Fusarium solani* showed 100% resistance to Amphotericin B

Most of the isolates in the study were sensitive to Itraconazole and were in the MIC range of 0.125 to 0.5µg/ml for Filamentous forms. 10.4% of *Asp.fumigatus* has shown MIC in resistant range. 100% resistance to itraconazole was seen among *Fusarium solani.*

Table 21: **Results of MIC by Agar Dilution method of Amphotericin B, Itraconazole and Voriconazole to Filamentous fungi:**

Species	No.	AMPHOTERICIN B			ITRACONAZOLE			VORICONAZOLE		
		S*	R	Mean MIC of Sensitive Isolates (µg/ml)	S*	R	Mean MIC of Sensitive Isolates (µg/ml)	S*	R	Mean MIC of Sensitive Isolates (µg/ml)
Asp.fumigatus	19	19 (100%)	-	0.25	18 (94.8%	1 (5.2%)	0.0313	19 (100%)	-	0.0313
Asp.flavus	8	8 (100%)	-	0.5	8 (100%)	-	0.25	8 (100%)	-	0.125
Asp.terreus	4	-	4 (100%)	-	4 (100%)	-	0.5	4 (100%)	-	0.25
Asp.niger	1	1 (100%)	-	0.125	1 (100%)	-	0.125	1 (100%)	-	0.125
Asp.nidulans	1	1 (100%)	-	0.5	1 (100%)	-	0.5	1 (100%)	-	0.125
Penicillium spp	3	3 (100%)	-	0.5	3 (100%)	-	0.125	3	-	0.125
Fusarium solani	2	-	2 (100%)	-	-	2 (100%)	-	2 (100%)	-	0.25
Rhizopus oryzae	3	3 (100%)	-	0.5	3 (100%)	-	0.25	3 (100%)	-	0.25
Absidia corymbifera	1	1 (100%)	-	0.25	1 (100%)	-	0.25	1 (100%)	-	0.25
Curvularia lunata	1	1 (100%)	-	0.5	1 (100%)	-	0.5	1 (100%)	-	0.5

Most isolates were sensitive to Amphotericin B, Itraconazole and Voriconazole by Agar dilution method of detecting MIC. 100% resistance was seen among *F.solani* and *Asp.terreus* for Amphotericin B, 100% of *F.solani* & 5.2% of *Asp.fumigatus* were resistant to Itraconazole.

Table 22: Results of MIC by E-test method of Amphotericin B and Itraconazole to Filamentous fungi:

Species	Number	Amphotericin B			Itraconazole		
		S	R	Mean MIC of Sensitive Isolates (µg/ml)	S	R	Mean MIC of Sensitive Isolates (µg/ml)
A.fumigatus	19	19 (100%)	-	0.0313	19 (100%)	-	0.0313
A.flavus	8	8 (100%)	-	0.5	8 (100%)	-	0.25
A.terreus	4	-	4 (100%)	-	4 (100%)	-	0.5
A.niger	1	1 (100%)	-	0.0313	1 (100%)	-	0.125
A.nidulans	1	1 (100%)	-	0.5	1 (100%)	-	0.5
Penicillium spp	3	3 (100%)	-	0.5	3 (100%)	-	0.125
F. solani	2	-	2 (100%)	-	2 (100%)	-	0.5
Rhizopus oryzae	3	3 (100%)	-	0.5	3 (100%)	-	0.25
Absidia corymbifera	1	1 (100%)	-	0.25	1 (100%)	-	0.25
Curvularia lunata	1	1 (100%)	-	0.5	1 (100%)	-	0.5

All isolates were sensitive to Amphotericin B and Itraconazole by Epsilometer test and MIC range was from 0.0313 to 0.5. 100% resistance to Amphotericin B seen among *Asp.terreus. F.solani* showed 100% resistance to both Amphotericin B and Itraconazole by E test.

Table 23: Results of Disc diffusion method of Amphotericin B and Itraconazole to filamentous fungi

Species	Number	Amphotericin B			Itraconazole		
		S (Zone >15mm)	SDD/I (Zone 13-14 mm)	R (Zone <12mm)	S (Zone >17mm)	SDD/I (Zone 14-16 mm)	R (Zone <13mm)
A.fumigatus	19	13 (68.4%)	3 (15.7%)	3 (15.7%)	14 (73.6%)	2 (10.5%)	3 (15.7%)
A.flavus	8	6 (75%)	1 (12.5%)	1 (12.5%)	5 (62.5%)	1 (12.5%)	2 (25%)
A.terreus	4	-	-	4 (100%)	3 (75%)	-	1 (25%)
A.niger	1	1 (100%)	-	-	1 (100%)	-	-
A.nidulans	1	1 (100%)	-	-	1 (100%)	-	-
Penicillium spp	3	3 (100%)	-	-	2 (66.6%)	1 (33.3%)	-
F. solani	2	-	-	2 (100%)	-	-	2 (100%)
Rhizopus oryzae	3	3 (100%)	-	-	3 (100%)	-	-
Absidia corymbifera	1	1 (100%)	-	-	1 (100%)	-	-
Curvularia lunata	1	1 (100%)	-	-	1 (100%)	-	-

S= SUSCEPTIBLE; SDD= SUSCEPTIBLE DOSE DEPENDENT; R= RESISTANT

68.4% *Asp.fumigatus* were sensitive, 15.7% were resistant and 15.7% were susceptible dose dependent. 75% of the *Asp.flavus* were sensitive, 12.5% were resistant and 12.5% were susceptible dose dependent. *Fusarium spp* and *Asp.terreus* showed 100% resistance to Amphotericin B. All other isolates were universally sensitive to Amphotericin B.

Of 19 *Asp.fumigatus*, 73.6% were sensitive,10.5% were susceptible dose dependent and 15.7% were resistant. Out of 8 *Asp.flavus* tested 62.5% were susceptible, 12.5% were susceptible dose dependent and 25% were resistant. All other isolates were sensitive to Itraconazole.

Table 24: Comparison of Disk diffusion, E Test and Agar dilution of Amphotericin B with Microdilution Reference Method to Filamentous fungi:

Species	Broth dilution(S)	Disk diffusion			E test		Agar Dilution	
		m	M	VM	M	VM	M	VM
A.fumigatus(19)	18	**3(15.6%)**	**2(10.4%)**	-	-	**1(5.2%)**	-	**1(5.2%)**
A.flavus(8)	8	**1(12.5%)**	**1(12.5%)**	-	-	-	-	-
A.terreus(4)	-	-	-	-	-	-	-	-
A.niger(1)	1	-	-	-	-	-	-	-
A.nidulans(1)	1	-	-	-	-	-	-	-
Penicillium spp(3)	3	-	-	-	-	-	-	-
Fusarium solani(2)	-	-	-	-	-	-	-	-
Rhizopus oryzae(3)	3	-	-	-	-	-	-	-
Absidia corymbifera(1)	1	-	-	-	-	-	-	-
Curvularia lunata(1)	1	-	-	-	-	-	-	-

m = minor error; M= Major error; VM= Very Major error

Interpretated as per the criteria given by A.Espinel –Ingroff et al:

Minor error(m): Shifts between susceptible and susceptible dose dependent or between resistant and susceptible dose dependent

Major error(M): Isolate resistant by other methods but susceptible by broth dilution

Very Major error(VM): Broth dilution shows resistance and others show as sensitive

Very major error of 5.2% in *A.fumigatus* was seen in both agar dilution and E test on comparing with broth dilution for amphotericin B.15.6% and 12.5% of minor error and 12.5% of major error each was seen among *A.fumigatus* and *A.flavus* respectively with disc diffusion method when compared to broth dilution method.

Table 25:Comparison of Disk diffusion, E Test and Agar dilution of Itraconazole with Microdilution Reference Method to Filamentous fungi:

Species	Broth dilution (S)	Disk diffusion			E test		Agar Dilution	
		m	M	VM	M	VM	M	VM
A.fumigatus(19)	17	**2(10.4%)**	**1(5.2%)**	-	-	**2(10.4%)**	-	**2(10.4%)**
A.flavus(8)	8	**1(12.5%)**	**2(25%)**	-	-	-	-	-
A.terreus(4)	4	-	-	-	-	-	-	-
A.niger(1)	1	-	-	-	-	-	-	-
A.nidulans(1)	1	-	-	-	-	-	-	-
Penicillium spp(3)	3	**1(33.3%)**	-	-	-	-	-	-
Fusarium solani(2)	-	-	-	-	-	-	-	-
Rhizopus oryzae(3)	3	-	-	-	-	-	-	-
Absidia corymbifera(1)	1	-	-	-	-	-	-	-
Curvularia lunata(1)	1	-	-	-	-	-	-	-

Very major error of 10.4% in *A.fumigatus* was seen in both agar dilution and E test on comparing with broth dilution for itraconazole.10.4% ,12.5% and 33.3% of minor error was seen among *A.fumigatus*, *A.flavus* and *Penicillium spp* respectively and 5.2% and 25% of major error was seen among *A.fumigatus* and *A.flavus* respectively with disc diffusion method when compared to broth dilution method.

Table 26: Comparison of Agar dilution for voriconazole with Microdilution Reference Method for Filamentous fungi:

Species	Broth dilution(S)	Agar Dilution	
		M	VM
A.fumigatus(19)	18	-	-
A.flavus(8)	8	-	-
A.terreus(4)	4	-	-
A.niger(1)	1	-	-
A.nidulans(1)	1	-	-
Penicillium spp(3)	3	-	-
Fusarium solani(2)	2	-	-
Rhizopus oryzae(3)	3	-	-
Absidia corymbifera(1)	1	-	-
Curvularia lunata(1)	1	-	-

There is no discrepancy seen between broth dilution and agar dilution for voriconazole in filamentous fungi.

Table 27: Prognostic interpretation of the follow up of study cases:

Follow Up	Proven IFI (n=35)	Probable IFI (n=44)	Possible IFI (n=121)	Total (%)
Cured	28(80%)	20(45.5%)	89(73.5%)	137(68.5%)
Relapse	2(5.7%)	16(36%)	20(16.5%)	38(19%)
Death	5(14.2%)	-	-	**5(2.5%)**
Lost To Follow Up	-	8(18.5%)	12(9.9%)	20(10%)

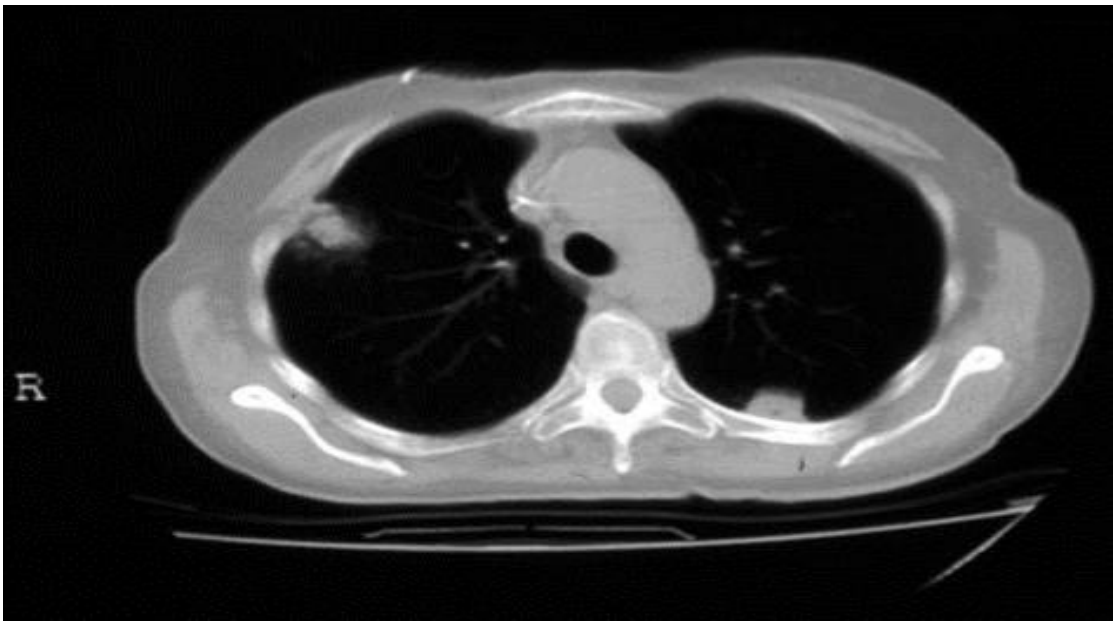
The overall mortality of 2.5% cases was seen in the study. All of them were proven IFI.

Mortality was seen among cancer patients on chemotherapy with neutropenia due to Candidemia and invasive aspergillosis, AIDS patient with a low CD4 count of <34cells/µl and in uncontrolled DM patients with Diabetic keto acidosis due to *Rhizopus oryzae* with grave complication like cavernous sinus thrombosis.

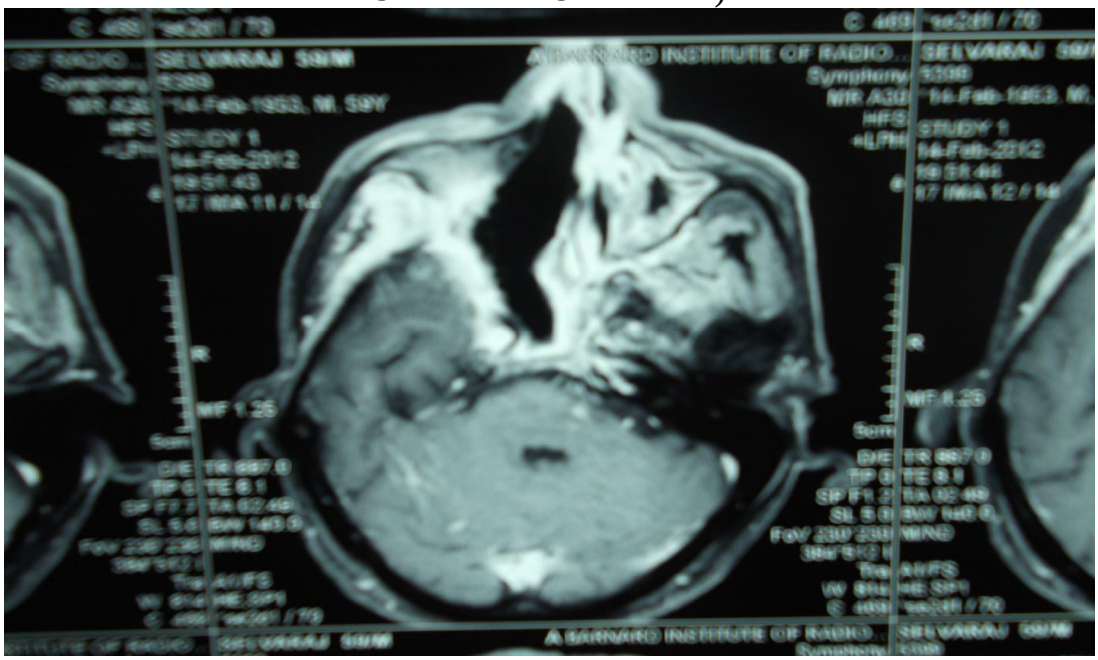
Relapse was commonly seen among probable IFI(36%). Cure rate of 73.5% was seen among possible IFI.

PATIENT WITH COMPLICATION OF CAVERNOUS SINUS THROMBOSIS

CT SCAN SHOWING HALO SIGN IN IA PATIENT

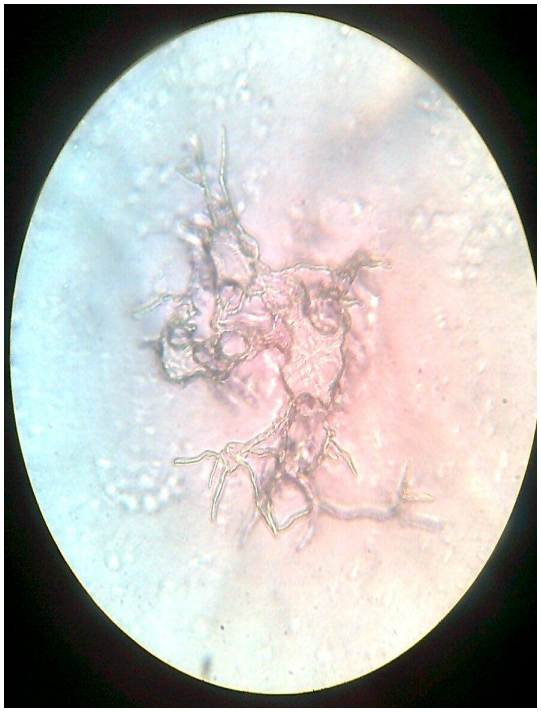


CT SCAN SHOWING CAVERNOUS SINUS THROMBOSIS(RIGHT SIDE PREDOMINANT)



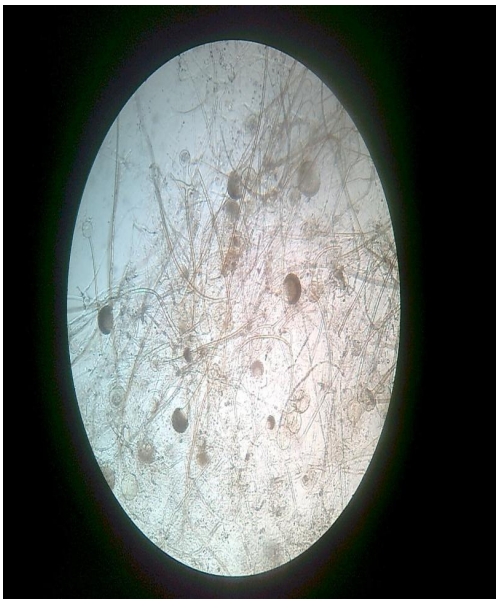
10% KOH- BROAD PAUCI SEPTATE HYPHAE WITH OBTUSE ANGLE BRANCHING

10% KOH- SLENDER PHAEOID SEPTATE HYPHAE

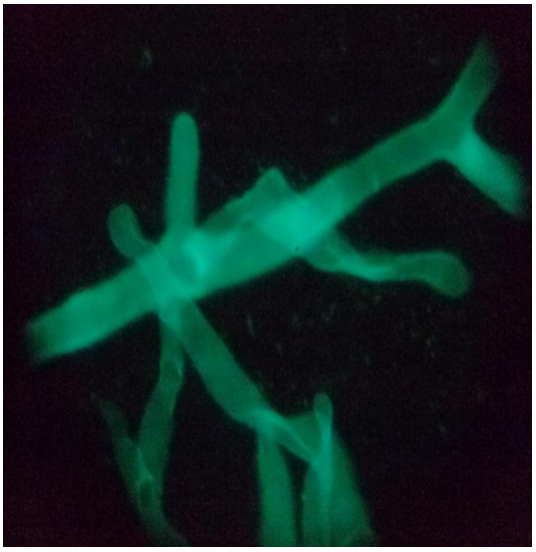


10% KOH- SLENDER SEPTATE HYPHAE WITH ACUTE ANGLE BRANCHING

10% KOH- BROAD PAUCISEPTATE HYPHAE WITH SPORANGIA

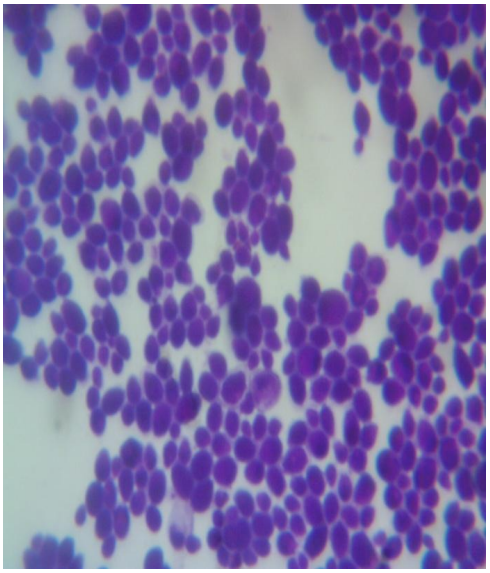


CFW SHOWING PAUCISEPTATE HYPHAE

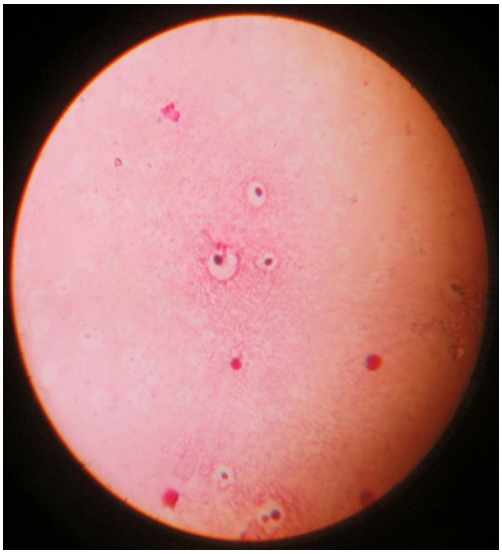


INDIA INK SHOWING CAPSULATED BUDDING YEAST

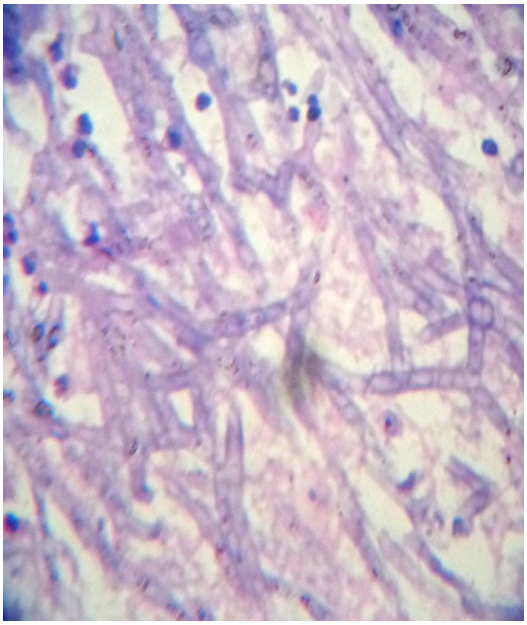
GRAM STAIN SHOWING BUDDING YEAST CELLS



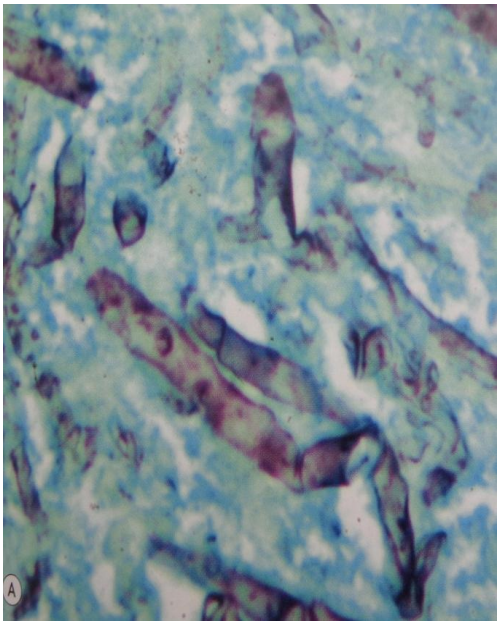
GRAM STAIN SHOWING CAPSULATED *CRYPTOCOCCUS SPP*



H&E SHOWING SEPTAL HYPHAL FORMS

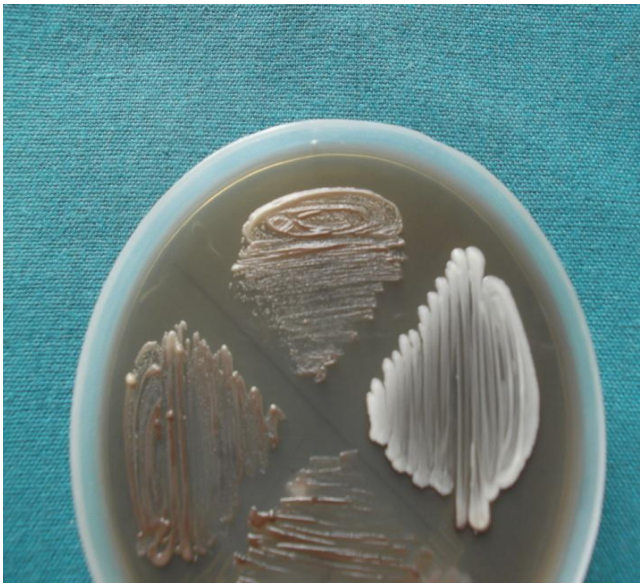


GMS SHOWING ASEPTATE HYPHAL FORMS



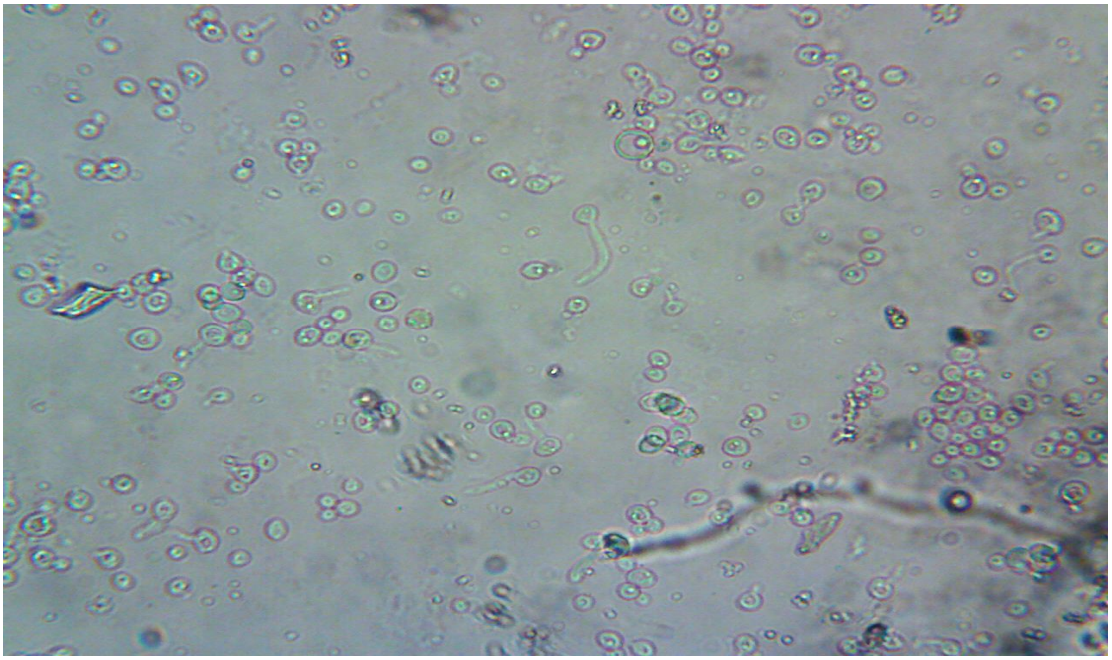
LAT CARD SHOWING CLUMPING FOR CRYPTOCOCCAL ANTIGEN

CAFFEIC ACID AGAR SHOWING BROWN COLOURED *CRYPTOCOCCUS* COLONIES



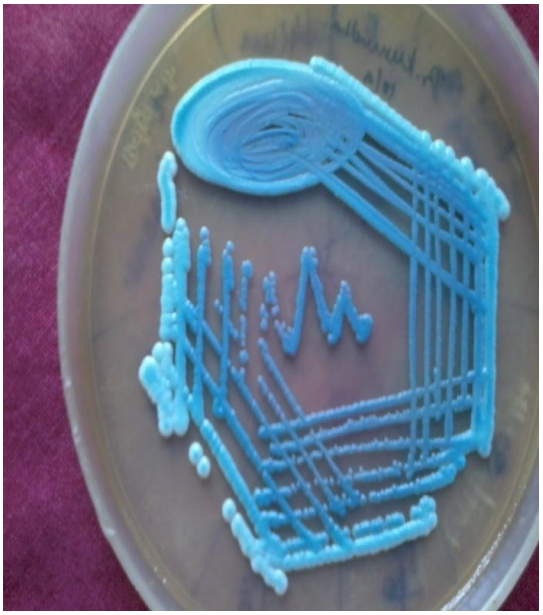
UREASE TEST POSITIVE

GERM TUBE TEST- *C.ALBICANS* SHOWING GERM TUBE

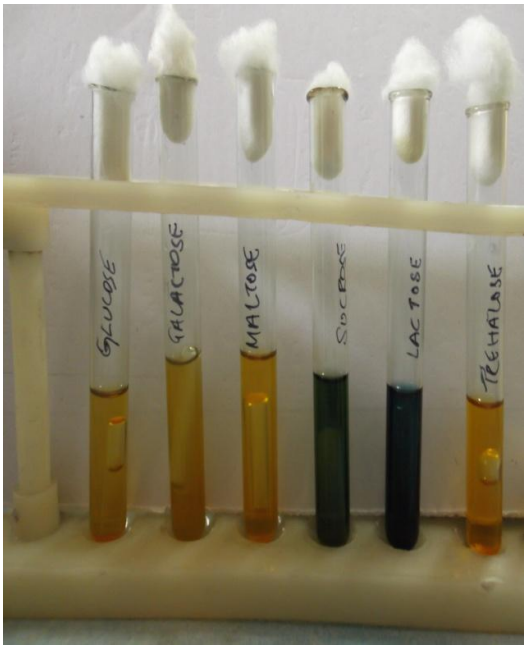


CHROMAGAR SHOWING LIGHT GREEN *C.ALBICANS*

CHROMAGAR SHOWING STEEL BLUE *C.TROPICALIS*

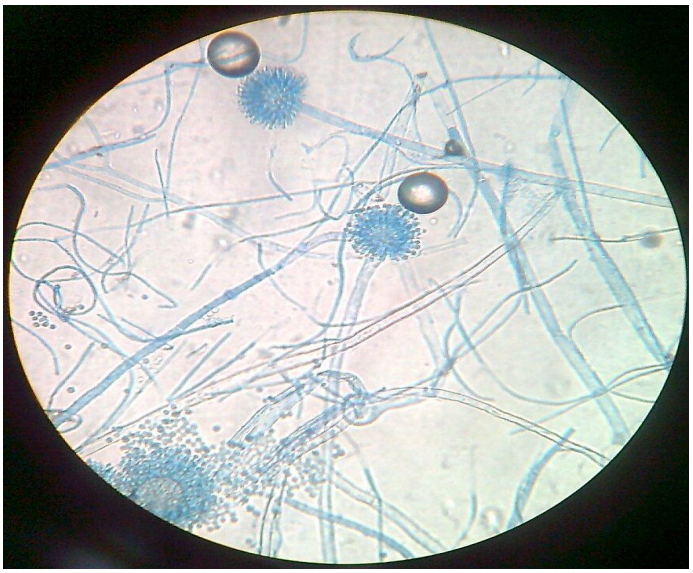


FERMENTATION OF *C.ALBICANS*

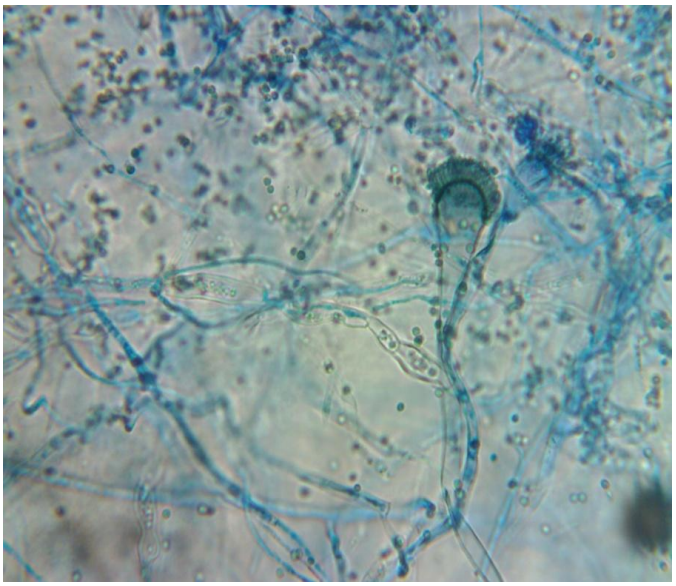


ASSIMILATION *OF C.ALBICANS*

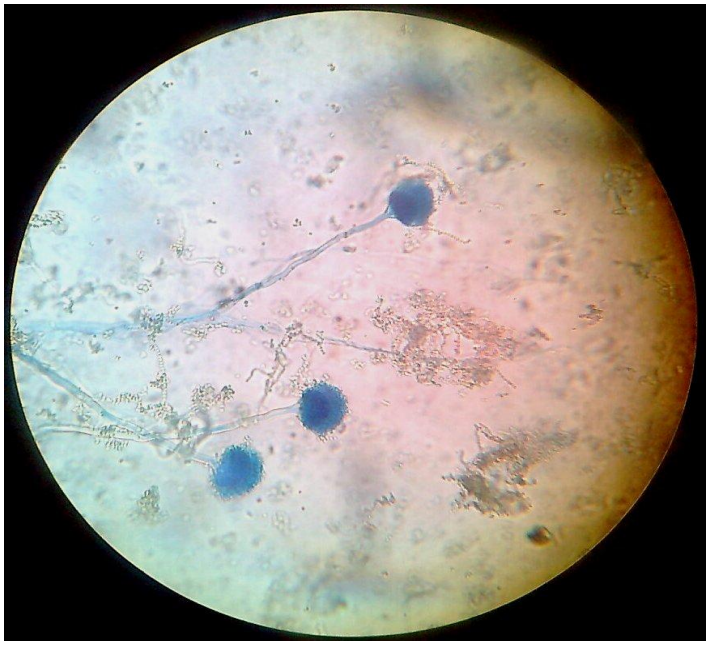
ASPERGILLUS FLAVUS



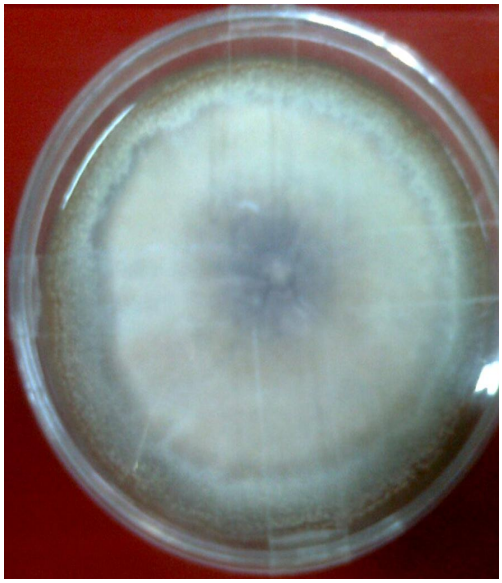
ASPERGILLUS FUMIGATUS



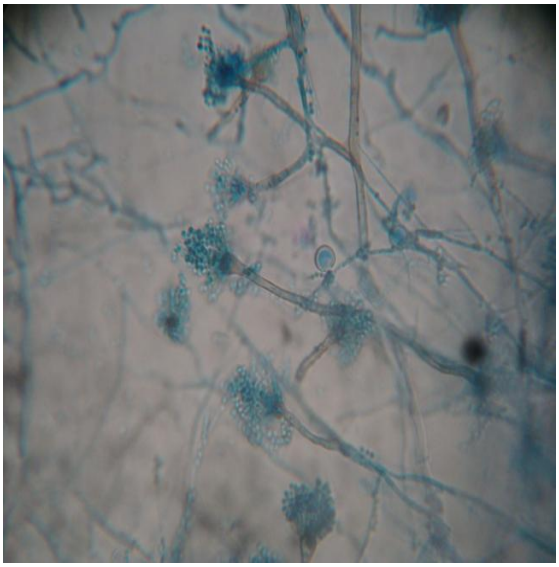
ASPERGILLUS TERREUS



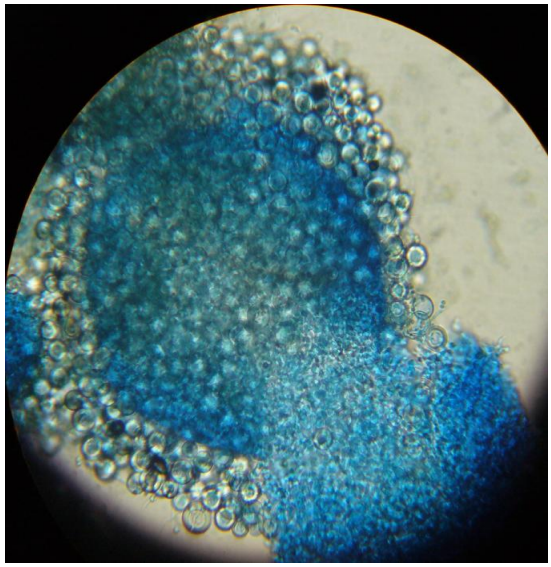
ASPERGILLUS NIDULANS- SDA PLATE SHOWING PRESENCE OF PURPLE PIGMENT ON THE REVERSE



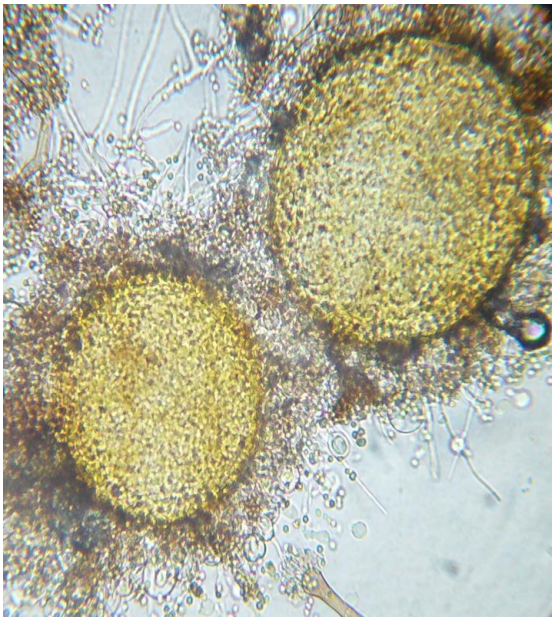
LPCB MOUNT SHOWING COLUMNAR CONIDIAL HEADS AND A SINGLE HULLE CELL

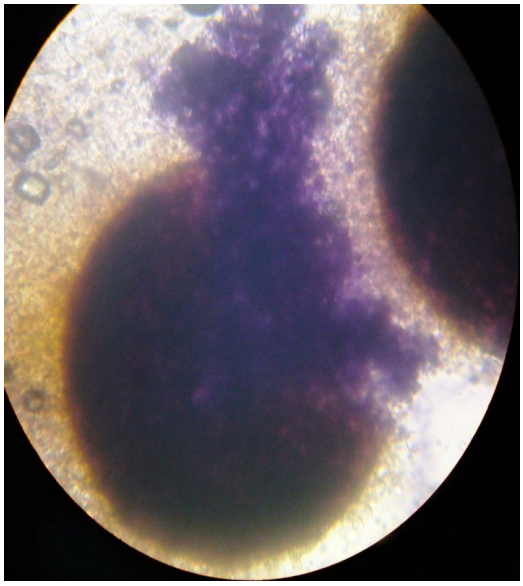


CLEISTOTHECIUM SURROUNDED BY HULLE CELLS

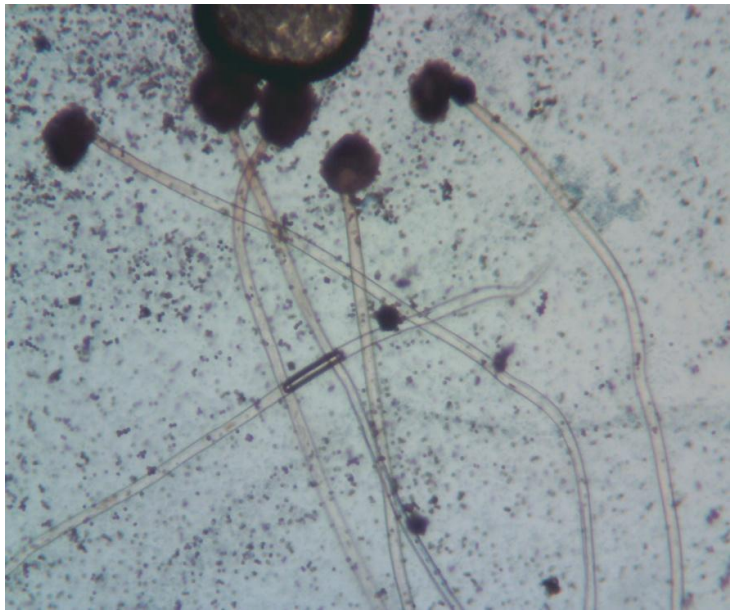


ADHESIVE TAPE PREPARATION WITH KOH SHOWING BROWN COLOURED CLEISTOTHECIA (LEFT) AND PURPLE COLOURED ASCOSPORES DUE TO REACTION WITH KOH (RIGHT)

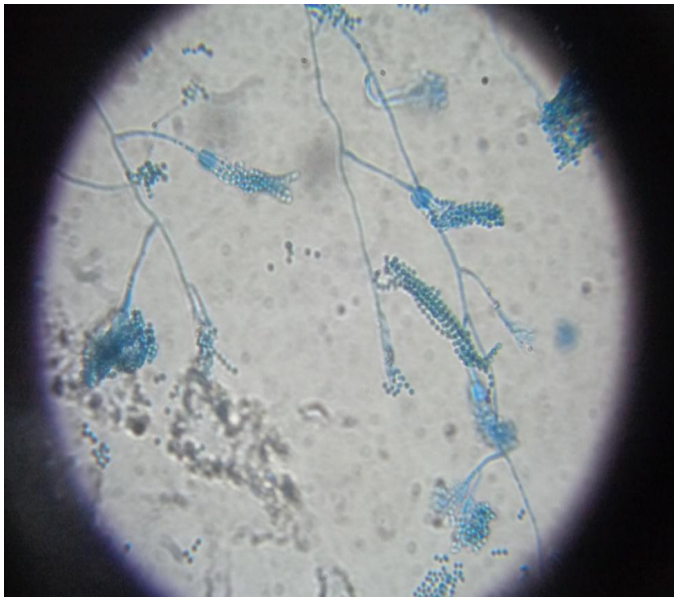




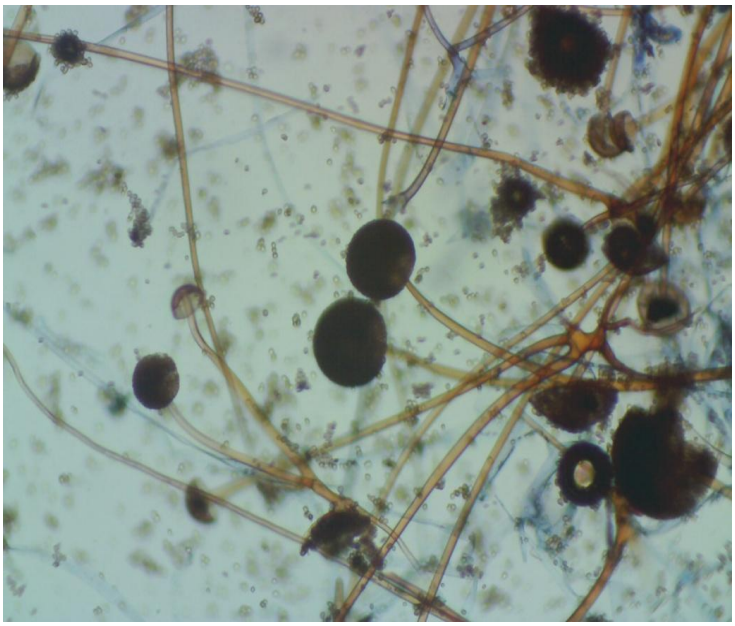
ASPERGILLUS NIGER



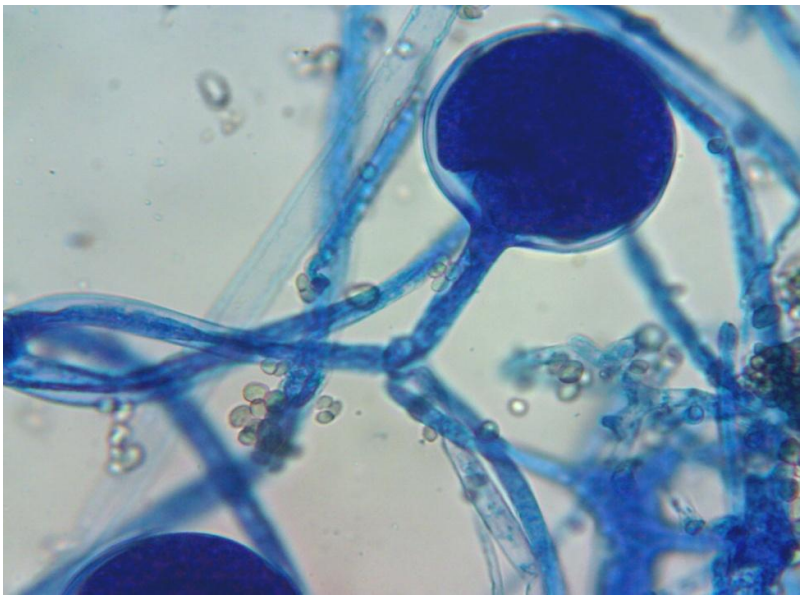
PENICILLIUM SPP



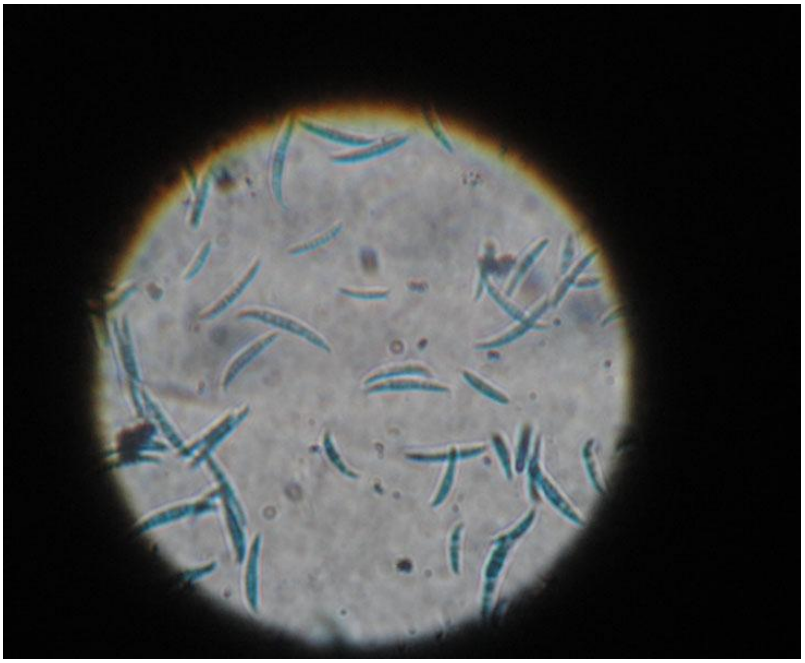
RHIZOPUS ORYZAE- UNBRANCHED SPORANGIOPHORE WITH NODAL RHIZOIDS



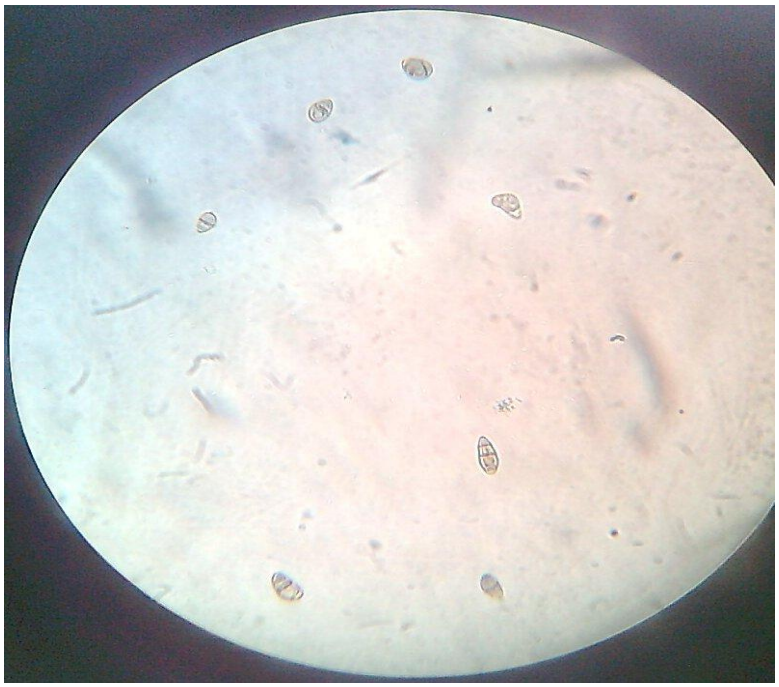
ABSIDIA CORYMBIFERA WITH APOPHYSIS



FUSARIUM SOLANI WITH PINK DIFFUSABLE PIGMENT
ON SDA TUBE

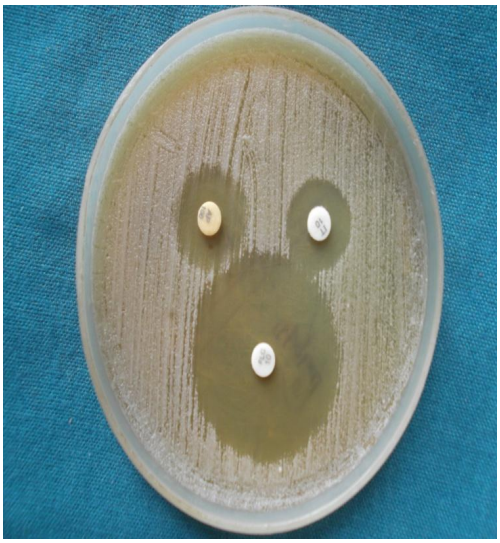


CURVULARIA LUNATA

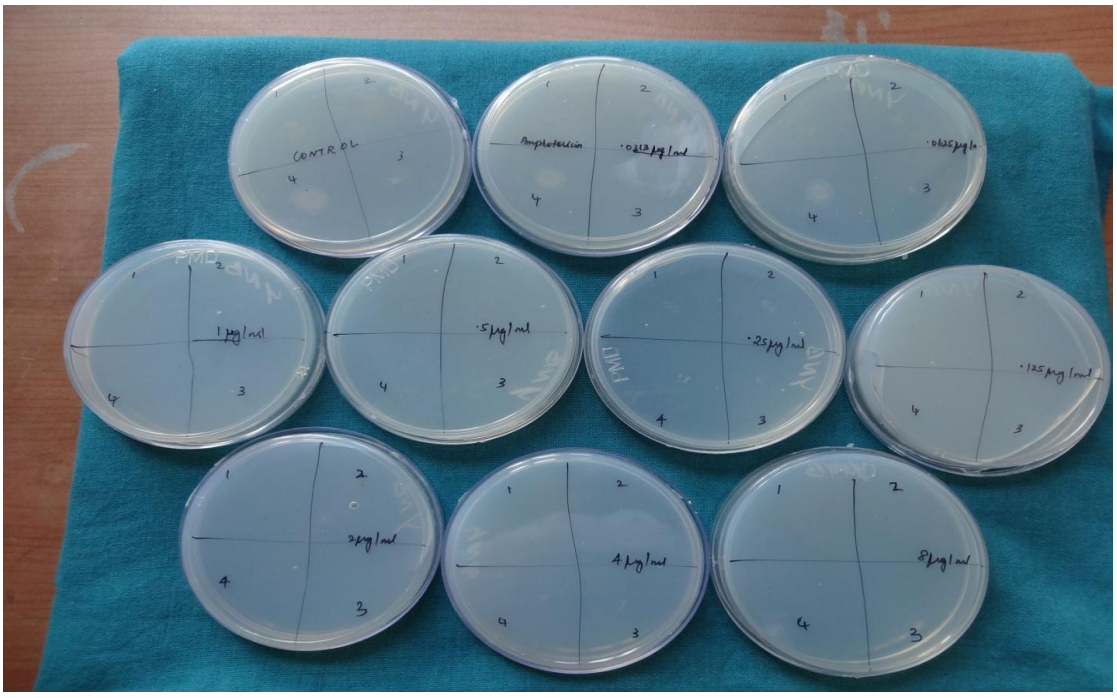


EXSEROHILUM ROSTRATUM – BLACK PIGMENTATION IN OBVERSE AND REVERSE

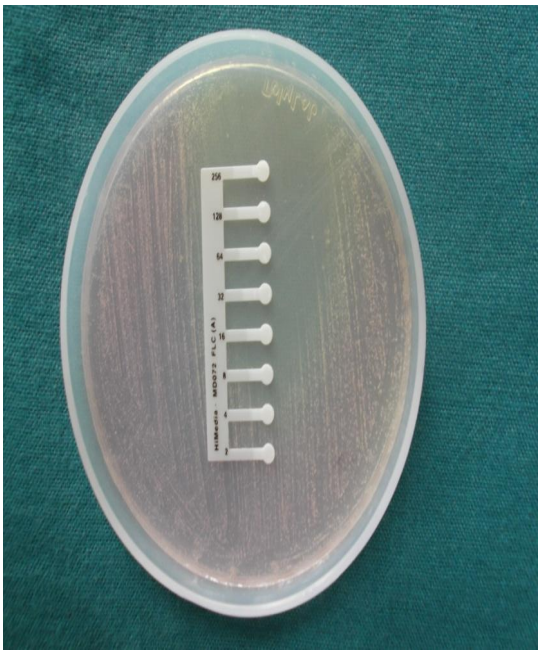
DISC DIFFUSION METHOD FOR YEAST LIKE AND FILAMENTOUS FUNGI



AGAR DILUTION METHOD OF AMPHOTERICIN B

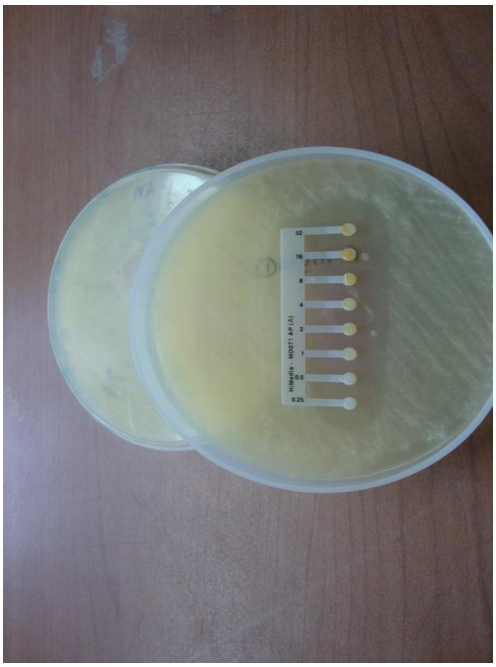


E TEST FLUCONAZOLE FOR *CANDIDA SPP*

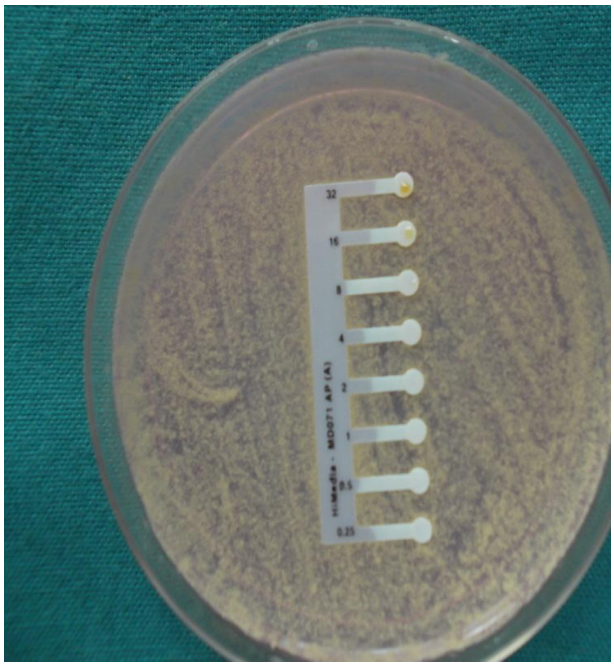


E TEST – ITRACONAZOLE FOR *A.NIGER*

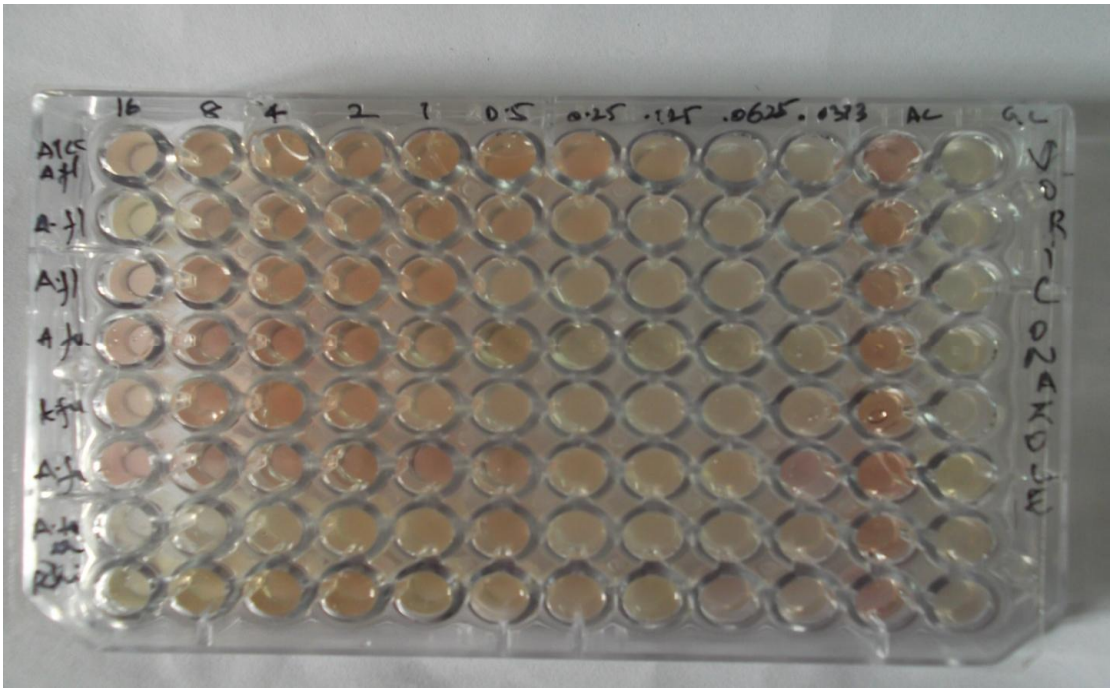
E TEST – AMPHOTERICIN B FOR *A.FLAVUS*



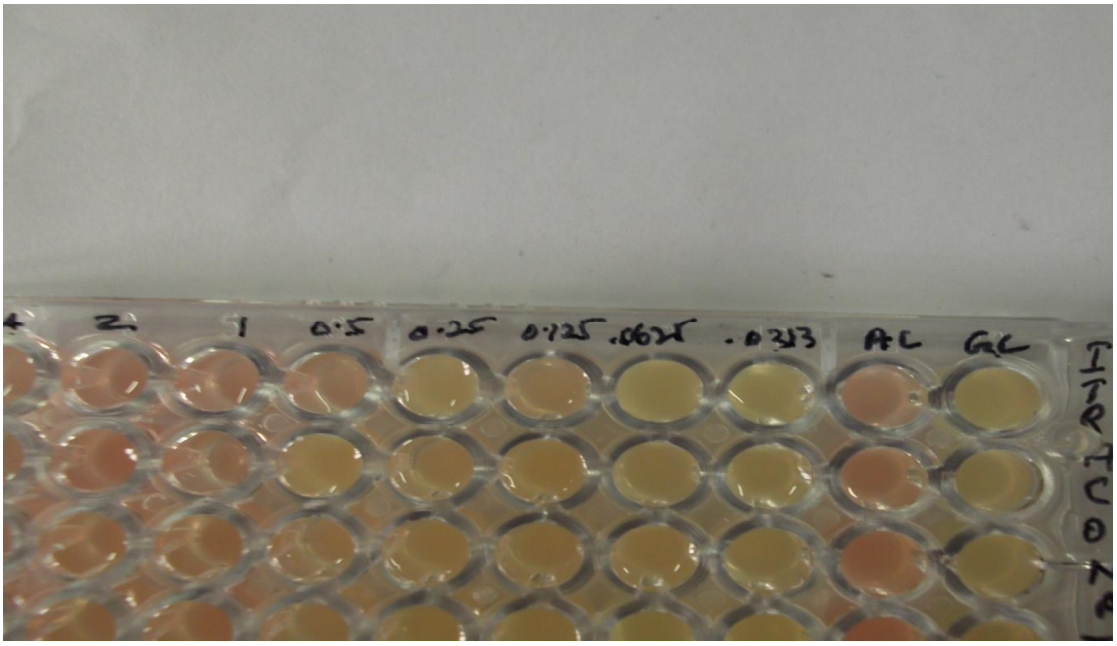
E TEST – AMPHOTERICIN B FOR *A.TERREUS*



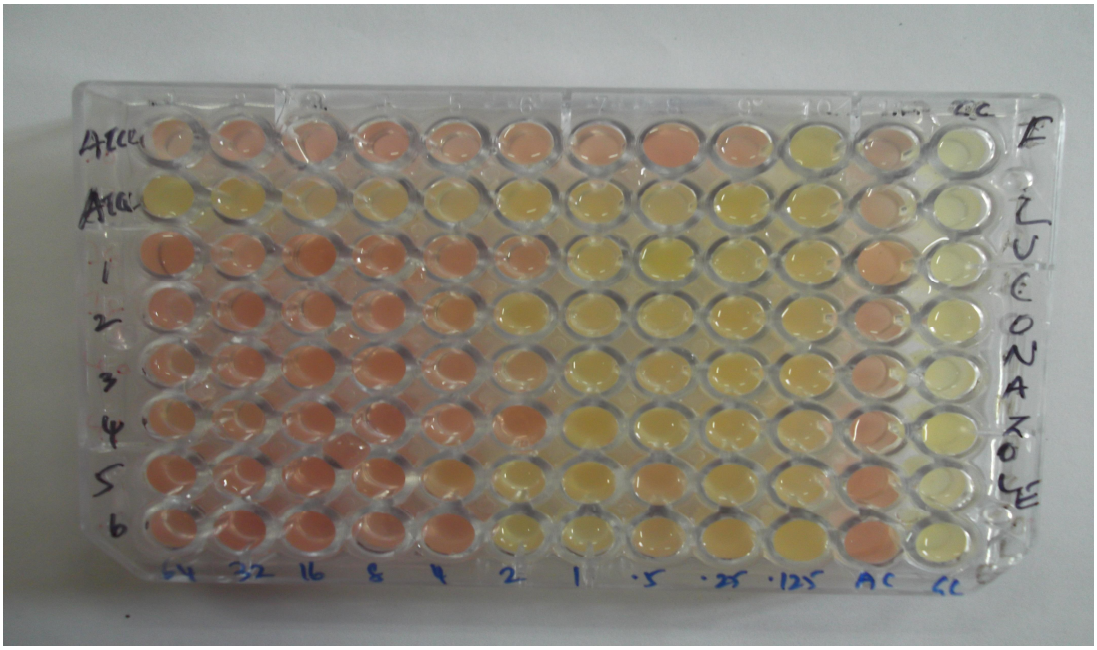
MIC BY BROTH MICRODILUTION OF VORICONAZOLE TO FILAMENTOUS FUNGI



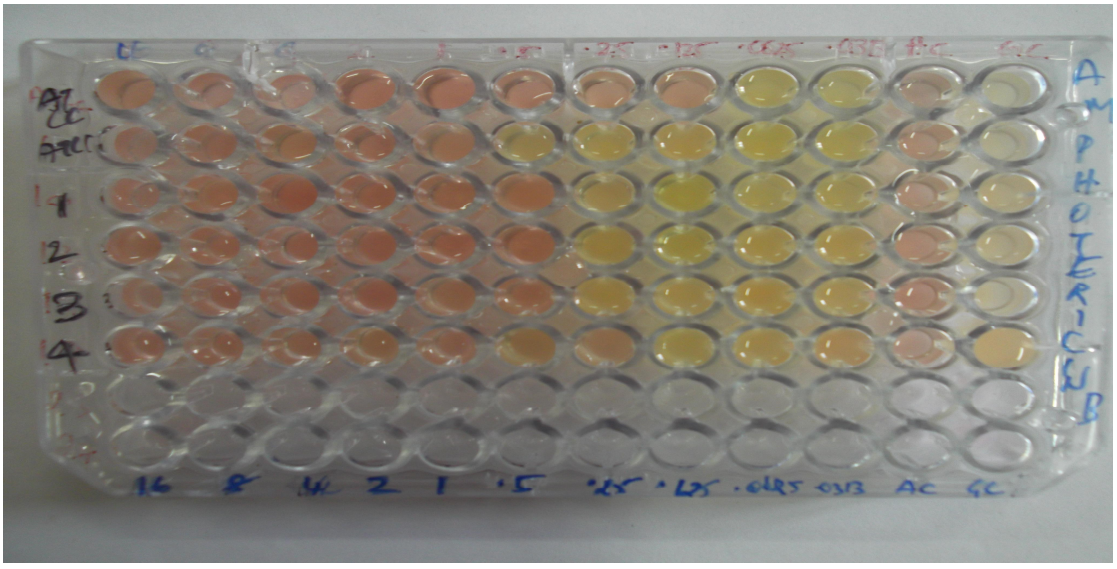
MIC BY BROTH MICRODILUTION OF ITRACONAZOLE TO FILAMENTOUS FUNGI



MIC BY BROTH MICRODILUTION OF FLUCONAZOLE *TO CANDIDA SPP*



MIC BY BROTH MICRODILUTION OF AMPHOTERICIN B *TO CRYPTOCOCCUS SPP*



DISCUSSION

DISCUSSION

Invasive Fungal Infection (IFI) is increasingly recognized among patients with immunocompromised state[3] .The importance is due to the morbidity and mortality caused by IFI. This study was conducted among 200 cases of immunocompromised patients with signs and symptoms suggestive of IFI. Patients selected under the study were those on chemotherapy for malignancy, renal transplant recipient on immunosuppressive therapy, patients with Acquired immune deficiency syndrome (AIDS) and Diabetes mellitus. The study was conducted at the Institute of Microbiology, Madras medical college & RGGGH, Chennai-3 for a period of one year from September 2011 to August 2012.

Most of the patients affected by IFI in this study (table 1) were in the age group of 31-40(26%), followed by equal number of distribution among 21-30 & 41-50 age group(22%). Among the study population, majority of the patients were males (73%). Wald et al, in his study on IFI among bone marrow transplant patients showed increased incidence among >40years age group with predominance noted among males(69.4%)[92] . Marr et al, has also made similar observation that IFI was more common among >40years(53%) with male(61%) predominance in stem cell transplant recipients[93] .Similar findings have been recorded by Kontoyiannis *et al* showed median age of the patients with IFI was 49years and 60% were males[94].

Among immunocompromised patients, median time for development of IFI was 1-10 years (table 2). As the duration of immunocompromised state increases, susceptibility to infection also increases. This finding is similar to Kontoyiannis et al,

who found a median duration for development of IFI in transplantation recipients of 135days [94] .

Majority of patients of IFI presented with symptoms presented with urinary tract and respiratory tract infections, therefore most samples collected were urine, sputum, bronchial wash and bronchoalveolar lavage (table 3). Pagano et al, studied patients with IFI and reported most common site for infection as lung(56%)[95] .

Among the 200 cases in our study, 79(39.5%) showed fungal growth. According to EORTC criteria among these 79 patients who showed fungal growth,17.5% were proven and 22% were probable and rest 60% were possible IFI (table 4). Siok-Ying lee et al reported a prevalence of IFI in hematological patients in Singapore of 30.8% proven, 48.7% probable and 20.5% possible IFI [96] . Kontoyiannis et al, studied on patients with IFI for a period of 5 years reported 56% of cases were proven and 44% were to be probable IFI[94] .

In our study, (table 4) 20.8% of proven IFI and 40.2% of probable IFI were among patients with uncontrolled diabetes mellitus (DM). Diabetes is a common metabolic disorder with significant morbidity and mortality, and is commonly considered as a risk factor of mycoses[3,97,98] .Usually in these patients, the most common fungal invasion are those of the urinary tract and the respiratory tract. The reason for increased incidence and susceptibility is due to impaired phagocyte function, such as adherence, chemotaxis, phagocytosis, and bactericidal activity in patients with poor metabolic control[3,99,100]. This finding in our study is similar to observations made by Jahromi and Khaksar et al,who isolated 28.4% of fungal infections among diabetic patients[3].

Next to DM, the second risk factor with major incidence of proven IFI was among malignant patients on chemotherapy (18.6%). Prolonged neutropenia in these patients acts as a predisposing factor, which suggests that the neutrophils have a major role in host defence against fungal infections. Host defence depends mostly on the phagocytic and oxidative activities of neutrophils, monocytes, and macrophagesThis finding of our study correlates with Jahromi and Khaksar et al who reported 14.8% among neutropenic patients[3] .

In our study among renal transplant recipients(n=65), Hepatitis C virus(26.1%) was the predominant predisposing factor for IFI. Fabrizi et al. showed that positive anti-HCV antibody status was a significant risk factor for fungal infection and death after renal transplantation with a relative risk of 1.79 and 1.56 respectively[101] .

In this study, Proven IFI is defined by Direct KOH mount and / or HPE positivity, fungemia in blood, India ink preparation or Antigen test positivity and culture positive from sterile site. Among proven IFI cases(n=35), 74.3% were detected by KOH/HPE(table 6).

Invasive aspergillosis was the most common IFI (16.5%), followed by invasive candidiasis (15%), hyalohyphomycosis due to *Penicillium spp* and *Fusarium spp (2.5%).* Zygomycosis and Cryptococccosis were responsible for 2% cases of IFI(table 7). The presence of hyphal elements in the direct KOH mount in all the specimens proven the clinical significance of our isolates. Our study finding is very similar to Wald et al [92] and Marr et al [102] who reported *Aspergillus spp* as the most common etiological agent isolated followed by *Candida spp.* But, our results are in contrast to the observation made by Kauffman et al[103] and Chakrabarti et al[1] who showed Invasive Candidiasis as the most common invasive mycotic infection across India.

Enoch et al [113] studied on IFI in Great Britain and Maertens et al [104] in Belgium showed similar findings.

Candida spp are usually colonizers, so if isolated their clinical significance needs to be defined. *Candida spp* from blood or on repeated isolation is taken as pathogen. In our study, among invasive candidiasis, *Candida albicans* was the most common organism(55.8%), followed by *Candida tropicalis*(14.7%), *Candida glabrata*(11.7%) and *Candida parapsilosis* (5.8%)(table 8).

B George et al [105] and Pagano et al [106] reported *Candida albicans* as most common isolate among *Candida spp* which is almost similar to the results of the present study.

The most common *Candida spp* isolated from blood culture were *C.albicans*(43%) and *C.glabrata*(28.5%)(table 10). The incidence of candidemia gives similar reports worldwide. In the study done by Tortorano et al[107], main agent of candidemia was *C. albicans*(58.5%) followed by *C.glabrata* (12.8%). A study by Trick et al reported *C. albicans* (59%) and *C.glabrata* (12%) from blood cultures of patients on chemotherapy with suspected IFI[108]. Richet et al reported *C. albicans* (53%) followed by *C.glabrata* (11%) in blood culture[109]. Similar to our study, all other studies also reported *C.albicans* as the leading pathogen followed by *C.glabrata* in blood culture.

Harvey et al[110] studied on transplant patients and reported an isolation rate of (58%) *C.albicans* followed by (25%) *C.tropicalis* and (15%) *C.parapsilosis.* In another study by Chakrabarti et al, on immunocompromised patients with suspected IFI the common isolates from blood culture were *C.albicans*(50%), *C.gulliermondii*(17%) and

C.tropicalis(8%)[111] . In our study we isolated *C.glabrata* as second leading pathogen in Candidemia whereas in above studies it was either *C.tropicalis* or *C.gulliermondii.*

In the present study from urine samples we isolated *C. albicans* (15.2%) as the most common isolate followed by *C. tropicalis* (8.4%), *C. glabrata* (3.2%) and *C.parapsilosis*(3.2%) (table 10). Study by Zarrin et al, among immunocompromised patients reported an isolation rate of *C. albicans* (53.3%), followed by *C. glabrata* (24.4%), *C. tropicalis* (3.7%), *C. krusei* (2.2%)[112].

The majority of the *Aspergillus spp* isolated were *Aspergillus fumigatus*(42.2%), followed by *Aspergillus flavus* (17.7%), *Aspergillus terreus*(8.8%), *Aspergillus niger* and *Aspergillus nidulans* (2.2%)(table 9) from respiratory tract specimens(table11). The number of non-*Aspergillus* moulds isolated were 12(15.2%) [(table 9) which included *Pencillium spp(6.6%), Rhizopus oryzae (6.6%), Fusarium solani(4.4%), Adsidia corymbifera(2.2%)*]. The dematiaceous mould involved in disease were *Curvularia lunata(2.2%), Exserohilum rostratum(2.2%)* and one unidentified dematatious fungi. Kontoyiannis et al [94] and Siok – Ying Lee et al[96] have shown in their studies that among *Aspergillus spp, Aspergillus fumigatus* was the most common species isolated from lung specimen. Their finding correlates with our study report(table 11).

Due to the life threatening nature of invasive fungal infections and reports of drug resistance, antifungal susceptibility testing(AST) for pathogens are very important.The primary objective of AST is to predict the impact of administration of the tested agent on the outcome of infection caused by the tested organism.

19 isolates of *Candida albicans*,5 of *C.tropicalis*,4 of *C.glabrata* and 2 of *C.parapsilosis* were taken for antifungal susceptibility testing for Amphotericin B, Itraconazole, Fluconazole and Voriconazole(table 12-15) by broth dilution, agar dilution, E test and disc diffusion methods. All Non *Candida albicans* (NCA) showed universal sensitivity to all antifungals tested except *C.glabrata* which showed complete resistance to Fluconazole alone. This may be due to intrinsic resistance exhibited by few strains of *C.glabrata*[42]

In our present study, *C.albicans* showed 5.2% resistance to Amphotericin B, Itraconazole and Fluconazole by broth dilution method. Agar dilution & E test gave equally similar results to Amphotericin B but 5.2% of very major error was seen between broth and agar dilution to itraconazole and fluconazole(table 16-19).

A study with *Candida spp* by Iatta et al using E test of Amphotericin B, Itraconazole, Fluconazole and voriconazole gave a comparable results similar to that of broth dilution method.[113] Barchiesia et al showed that broth microdilution and agar dilution methods are equally good in testing *Candida spp*.[114]

But the present study showed 10.4% resistance to amphotericin B, 5.2% to itraconazole and 10.4% to fluconazole was reported in *C.albicans* by disc diffusion(DD) method. A discrepancy of 5.2% was seen between DD and broth dilution procedure(table 16-19).

In a study by Goel et al which showed 95.53% of the *Candida* isolates to be sensitive to fluconazole, a discrepancy between DD method and broth micro dilution method was noted in 4.47% strains of *C.albicans*.[115]

All the 4 *Cryptococcus neoformans* isolates in this study were tested by broth dilution, agar dilution and E test showed 100% sensitivity to Amphotericin B, Fluconazole and Voriconazole(table 16-19). AST to Itraconazole was not done since it does not cross blood brain barrier. As disc diffusion method is not recommended for *Cryptococcus spp* it was not done. Archibald et al, studied the susceptibility pattern of *C.neoformans* and reported 100% sensitivity to various antifungal agents.[116]

In our study,19 isolates of *Aspergillus fumigatus,* 8 of *A.flavus*,4 of *A.terreus*,3 each of *Penicillium spp* and *Rhizopus oryzae*,2 of *Fusarium solani*, and 1 each of *A.niger, A.nidulans, Absidia corymbifera* and *Curvularia lunata* were taken for antifungal susceptibility testing for Amphotericin B, Itraconazole and Voriconazole (table 20-23). All the tested isolates were universally sensitive to Voriconazole by broth dilution method and agar dilution method with no discrepancy (table 26).

All isolates were mostly sensitive to Amphotericin B by broth dilution method except 5.2% of *A.fumigatus* which showed resistance. But 100% sensitivity to amphotericin B was seen by agar dilution and E test methods for all filamentous fungi. Therefore a discrepancy of 5.2% was seen in agar dilution & E test on comparing with broth dilution method((table 24). All isolates of *A.terreus* and *Fusarium solani* showed 100% resistance to Amphotericin B(table 20), since they exhibits intrinsic resistance to the drug[42] .On testing with itraconazole, the results obtained by agar dilution & E test method was comparable to that of microbroth dilution method for all tested isolates but discrepancy of 10.4% was observed(table 25).

In a study carried out by Theres et al., it was observed that agar dilution method is more easy and cost effective for the susceptibility testing of filamentous fungi over broth dilution method[117].

Filamentous fungi isolated in the study showed 68.4% & 73.6% sensitivity was seen to Amphotericin B and itraconazole respectively by disc diffusion method. The percentage of major (15.6%) error obtained by using Amphotericin B was found to be much higher when compared to itraconazole(5.2%) by disc diffusion method . Therefore, Amphotericin B discs should be used with caution in testing filamentous fungi (table 24,25).

Soraino et al., showed the advantage of disc diffusion method over other methods such as broth dilution or E test for voriconazole susceptibility testing of *Aspergillus*. Only few reports are available on comparative analysis of disc diffusion and broth dilution method for susceptibility testing of filamentous fungi[118].

Although broth dilution method is the recommended standard method suggested by CLSI, this method is associated with some technical drawbacks such as cumbersome procedure, time consuming, technically demanding with poor end point precision particularly when fungistatic agents such as azoles are tested. While on the other hand disc diffusion method is easy to perform, rapid and cost effective, so they are used for the routine antifungal susceptibility testing of filamentous fungi in the clinical laboratory. But Amphotericin B discs should be used with caution[119].

Lesser the time taken for results of AST better would be the therapeutic outcome of infections caused by fungal pathogens.

Follow up was done for all the patients included in the study but during the study period few patients expired. Overall mortality rate was 2.5%(table 27). Most of them were from proven IFI. One the major risk factor for mortality in the study was uncontrolled DM with ketoacidosis (40%) due to extension of *Rhizopus oryzae* from

nasal mucosa to turbinate bone into the brain causing infarct and cavernous sinus thrombosis. The other major risk factor was patients on chemotherapy (40%) due to Candidemia caused by drug resistant *C.albicans* & disseminated aspergillosis due to *Asp.fumigatus*. mortality was also seen in AIDS patient(20%) with a low CD4 count of 34 cells/µl.

United states data shows that in 1980 IFI was responsible for 828 deaths and was the 10th most predominant cause of fatal infection. However in 1997 the number of mycosis related deaths had risen to 2370 and was the seventh most prevalent terminal infectious disease [120,121] .

A study done by Martino et al[93] and Kontoyiannis et al[94] on patients with Candidemia showed a mortality rate of 8.3% and 11.5% respectively. Mortality rate of 36% due to Aspergillosis was reported by Jantunen et al [122] .

Strong suspicion, meticulous specimen collection & preparation and further studies with a long period of follow up and a large study population are required to analyze the impact of fungi in etiopathogenesis of invasive fungal infections.

SUMMARY

SUMMARY

- 200 patients who complied with the inclusion criteria were included under the study. The most common age group of the study population was 31 to 40 years (26%) and males were predominant(73%).
- Incidence of IFI occurs mostly after a prolonged period of immunosuppresion of 1-10 years(77%)
- Most frequently encountered samples in this study were urine (29.5%) and sputum (12.5%).
- Most of the IFI cases fall under the category of Possible IFI(60.5%)
- 17.5% were under the category of Proven IFI, mostly identified by KOH and/ or HPE (74.2%).
- The rate of invasive fungal infection (proven and probable) in this present study was 39.5%.
- Among the cases which showed fungal growth, Diabetes mellitus was the most predominant risk factor encountered in 33.5% of cases.
- Among the 65 renal transplant recipients under the study, 26.1% were HCV positive and in them 11.7% had proven IFI,5.88% had probable IFI and 21.5% had possible IFI.
- Etiological agents were identified in 79(39.5%) of infected patients.
- Aspergillosis was most commonly encountered(16.5%), followed by Candidiasis(15%) as etiological agents of IFI.

- Among filamentous fungi *A.fumigatus* (42.2%) was the most common pathogen isolated.
- Among Yeast like fungi *C.albicans* (55.8%) was the most common pathogen isolated.
- All isolates were sensitive to Voriconazole by broth microdilution and agar dilution
- Antifungal susceptibility testing by microbroth dilution to yeast like fungi, showed that 5.2% of *C.albicans* was resistant to amphotericin B, itraconazole and fluconazole.
- All non *Candida albicans* isolates were sensitive to all antifungals except C.*glabrata* which showed resistance to fluconazole.
- No discrepancy was noted between microbroth dilution, agar dilution and E test for Amphotericin B. But a major error of 5.2% was seen between broth dilution and disc diffusion test for *Candida spp*.
- Discrepancy of 5.2% was seen between microbroth dilution and agar dilution for itraconazole and fluconazole.
- 100% agreement was seen with microbroth dilution and agar dilution for voriconazole to yeast like fungi.
- *Cryptococcus neoformans* was mostly isolated from AIDS patients and were 100% sensitive to all antifungals.

- *Asp.fumigatus* showed 5.2% and 10.4% of resistance to Amphotericin B and itraconazole respectively by microbroth dilution method.
- Discrepancy of 5.2% and 10.4% was seen on comparing microbroth dilution with agar dilution & E test to Amphotericin B and itraconazole respectively to *Asp.fumigatus* in filamentous fungi.
- Discrepancy of 10.4% and 5.2% was seen on comparing microbroth dilution with disc diffusion to Amphotericin B and itraconazole respectively in filamentous fungi.
- 100% agreement was seen between broth dilution and agar dilution for voriconazole for filamentous fungi.
- Mortality rate encountered was 2.5% and all the cases had proven IFI.

CONCLUSION

CONCLUSION

The present study was done on 200 suspected invasive fungal infection(IFI) cases which showed that majority of patients were in the age group of 31 to 40 years and they were predominantly males. Most of them had urinary and respiratory tract infections following a prolonged period of immunosuppresion ranging from 1-10 years.

On categorization 17.5% had proven IFI, which was identified in majority of the cases HPE and / or KOH. Uncontrolled diabetes mellitus was the comorbid risk factor for IFI.

Etiological agent was identified in 39.5% of infected patients. Aspergillosis caused by *Asp.fumigatus* was the leading cause for IFI followed by Candidiasis caused by *C.albicans*.

Antifungal susceptibility test by microbroth dilution, agar dilution, E test and disc diffusion methods to amphotericin B, itraconazole, fluconazole and voriconazole showed equally similar results for most of the isolates. Few discrepancies were seen among *A.fumigatus* and *C.albicans* mostly by disc diffusion method.

Proven IFI was life threatening and mortality was seen 2.5% of cases.

Due to the evolving nature of the epidemiology of invasive fungal infections in immunocompromised persons, continued research and surveillance are essential to optimize the prevention and therapy of IFI in them.

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AST	Antifungal susceptibility test
BSI	Blood stream infections
CLSI	Clinical and laboratory standards institute
CMV	Cytomegalovirus
CT	Chemotherapy
CXR	Chest X ray
DD	Disc diffusion
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
E TEST	Epsilometer test
ELISA	Enzyme Linked Immunosorbent Assay
GMS	Gomori Methenamine Silver
H&E	Haematoxylin and eosin
HCV	Hepatitis C virus
HPE	Histopathological examination
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IFI	Invasive fungal infection
KOH	Potassium hydroxide mount
LAT	Latex agglutination test
M	Minor error
M	Major error
VM	Very Major error
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MOPS	3N-Morpholino Propane Sulphonic Acid
MRI	Magnetic resonance imaging
NCA	Non Candida albicans
PAS	Periodic Acid Schiff
PCR	Polymerase chain reaction
PDA	Potato Dextrose agar
PNA FISH	peptide nucleic acid fluorescence in situ hybridization
RPMI	Rosewall Park Memorial Institute
RT	Renal transplantation
R	Resistant
S	Susceptible
SDD	Susceptible Dose Dependent

APPENDIX I

A. STAINS AND REAGENTS

1) 10% POTASSIUM HYDROXIDE SOLUTION:

Potassium hydroxide	10 gm
Glycerol	10 ml
Distilled water	80 ml .

2) LACTOPHENOL COTTON BLUE STAIN

Lactic acid	20 ml
Phenol	20 ml
Cotton blue (dye)	0.5 g
Glycerol	40 ml
Distilled water	20 ml

3) GRAM STAINING

Methyl violet (2%)	l0g Methyl violet in l00ml absolute alcohol in 1litre of distilled water (primary stain)
Grams Iodine	l0g Iodine in 20g KI (fixative)
Acetone	Decolourising agent
Carbol fuchsin	1% Secondary stain

4) INDIA INK PREPARATION- NEGATIVE STAINING:

India ink	150ml
Merthiolate (1:1000)	3ml
Tween 80(1:10000)	0.1ml

5) NIGROSIN STAIN:

Nigrosin granules	10gm
Formalin (10%)	100ml

6)CALCOFLUOR WHITE STAIN:

CFW M2R	100mg
Evans blue	50mg
Distilled water	100ml

B. MEDIA USED

1)SABOURAUD DEXTOSE AGAR WITH ANTIBIOTICS:

Ingredient	gm/ltr
Peptone	10 gms
Dextrose	40 gms
Agar	20 gms
Distilled water	1000 ml
Gentamicin	20 mg

Final pH was adjusted to 5.6.

The above ingredients were reconstituted in one litre of distilled water. Dissolve the powder in distilled water by boiling. Gentamicin is added to the boiling medium. The medium was then removed from heating, mixed well and then dispersed in tubes and autoclaved at 121^0 C for 15 minutes and the final pH was adjusted to 5.6. The tubes were cooled in slanted position and later the slants were stored in refrigerator.

2) YEAST NITROGEN BASE AGAR MEDIUM(DEHYDRATED NEDIA, (Himedia, MUMBAI)

INGREDIENTS	GRAMS/L	INGREDIENTS	GRAMS/L
Ammonium sulphate	5.00	Thiamine hydrochloride	0.004
L-Histidine hydrochloride	0.01	Boric acid	0.0005
DL-Methionine	0.02	Copper sulphate	0.00004
DL-Tryptophan	0.02	Potassium iodide	0.0001
Biotin	0.000002	Ferric chloride	0.0002
Calcium pantothenate	0.00004	Manganese sulphate	0.0004
Folic acid	0.000002	Sodium molybdate	0.0002
Inositol	0.02	Zinc sulphate	0.0004
Niacin	0.0004	Monopotassium phosphate	1.00
Para amino benzoic acid	0.0002	Magnesium sulphate	0.50
Pyridoxine hydrochloride	0.0004	Sodium chloride	0.10
Riboflavin	0.0002	Calcium chloride	0.10

Dissolve 6.7 gms of the media in 100 ml of distilled water. Sterilise by filteration and store at 4^0C.

3) MUELLER HINTON AGAR:

Beef infusion : 300 ml

Casein hydrosylate : 17.5 gm

Starch : 1.5 gm

Agar : 10 gm

Distilled Water : 1000 ml

pH : 7.4

Sterilize by autoclaving at 121^0 C for 20 minutes.

4) RPMI 1640(ROSEWALL PARK MEMORIAL INSTITUTE) MEDIA:

RPMI medium : 10.4 gm

MOPS buffer : 34.43 gm

Dissolve powered medium in 900 ml distilled water. Add MOPS to a final concentration of 0.165 mol/L and stir until dissolved. While stirring, adjust the pH to 7.0 at 25^0 C. Add additional water to bring medium to a final volume of 1000 ml. Filter sterilize and store at 4^0 C.

5) POTATO DEXTROSE AGAR:

Ingredient	gm/ltr
Potato	200 gms
Dextrose	20 gms
Agar	20 gms
Water	1 Litre

Boil 200 g of potatoes in 1 litre of water for 60 minutes. Squeeze as much as pulp as possible through a fine sieve. Add agar and boil till it dissolves. Add dextrose and make upto 1 litre. Dispense in required amounts taking care to keep the solids in suspension. Autoclave at 115^0 C for 30 minutes. Cool to 50^0 C and pour into petridishes.

6)CAFFEIC ACID FERRIC CITRATE MEDIUM

Ingredient	gm/ltr

Yeast extract	2
Dextrose	5
Ammonium sulphate	5
Dipotassium phosphate	5
Magnesium sulphate	0.7
Caffeic acid	0.18
Ferric citrate	0.02
Agar	20

pH 6.5± 0.2

Suspend 33.7 grams in 1 litre of distilled water. Heat until the solution boils to dissolve the contents completely. Sterilize by autoclaving at 121^0C for 15 minutes. Cool to 55^0C, add chloramphenicol 50µg/ml and dispense in plates/ tubes

7)CHROMAGAR CANDIDA MEDIUM:

Ingredient	gm/ltr
Peptone	15gms
Yeast extract	4gms
Dipotassium hydrogen phosphate	1gm
Chromogenic Mixture	7.22gms
Chloramphenicol	0.50gms
Agar	15gms

pH 6.3± 0.2 at 25^0C

42.72 gms of media suspended in 1000ml distilled water and heated to boiling for the medium to dissolve completely. Not to be autoclaved. Cool to 50^0C and dispense in sterile petriplates.

8)BRAIN HEART INFUSION AGAR(BHIA):

Brain Heart Infusion	37 gms
Glucose	20gms
L-Cysteine hydrochloride	1 gm
Agar	20gms
Distilled water	900ml

Gentamicin : 20 mg

Final pH was adjusted to 5.6.

Ingredients are dissolved by boiling and dispensed in screw capped tubes and autoclaved at 121^0C for 15 minutes.

9)BIPHASIC MEDIUM:

BHIA plus broth

BHIA is sterilised first and bottles are kept at slanting position, then BHI broth added aseptically to cover 40% of agar in standing position.

10) **Blood agar (5% sheep blood agar)**

Peptone	-	l0g
Nacl	-	5g
Distilled water	-	1 Ltr
Agar	-	10g

Dissolve ingredients in distilled water by boiling, and add 5% sheep blood (sterile) at 55℃ adjust pH to 7.4.

C. MEDIA REQUIRED FOR BIOCHEMICAL IDENTIFICATION

1)CHRISTENSEN'S UREASE TEST MEDIUM

Peptone	1g
Sodium chloride	5g
Dipotassium hydrogen phosphate	2g
Phenol red	6ml
Agar	20g
Distilled water	1 ltr
10% sterile solution of glucose	l0ml
Sterile 20% urea solution	l00ml

Sterilize the glucose and urea solutions by filtration. Prepare the basal medium without glucose and urea, adjust to pH 6.8-6.9 and sterilize by autoclaving in a flask at 121°C for 30min. Cool to about 50°C, add the glucose & urea, and tube the medium as slopes.

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No : 04425305301
Fax : 04425363970

CERTIFICATE OF APPROVAL

To
Dr. K.S. Kumudha Valli
PG in MD Microbiology
Madras Medical College, Chennai -3

Dear Dr. K.S. Kumudha Valli

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled " A study on Invasive Fungal Infections among Immunocompromised patients in a tertiary Care Hospital " No. 29092011

The following members of Ethics Committee were present in the meeting held on 27.09.2011 conducted at Madras Medical College, Chennai –3

1. Dr. S.K. Rajan MD	-- Chairperson
2. Dr. V. Kanagasabai MD Dean, Madras Medical College, Chennai -3	-- Deputy Chairman
3. Prof. R. Sundaram MD Vice Principal, Madras Medical College, Chennai -3	-- Member Secretary
4. Prof. R. Nandhini MD Director , Inst. of Pharmacology, MMC , Ch-3	-- Member
5. Prof. Pregna B. Dolia MD Director , Inst. of Biochemistry, M M C, Ch-3	-- Member
6. Thiru . Ulaganathan Administrative Officer, M M C, Ch-3	-- Layperson
7. Thiru. S. Govindasamy BA BL	-- Lawyer
8. Tmt. Arnold Saulina .MA., MSW	-- Social Scientist

We approve the Proposal to be conducted in its presented from

Sd/ Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study , any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report.

Member Secretary, Ethics committee

APPENDIX III- PROFORMA

S.no: IP no:

Name : Ward:

Age: Sex:

Occupation: Address:

PRESENTING COMPLAINTS: Duration

H/O Diabetes mellitus

H/O Cancer

H/O immunosuppressive therapy

H/O Uremia/Chronic kidney disease

H/O Prior transplant surgery

H/O Prior antibiotic therapy

Previous H/O IFI

H/O dialysis

H/O iron therapy

PHYSICAL EXAMINATION:

BIOLOGICAL PARAMETERS:

Blood sugar (fasting and post prandial)

S.urea:

S.creatinine:

OTHER INVESTIGATIONS:

Complete hemogram:

Total WBC count

Differential count:

ESR:

Renal function test:

Liver function test:

CD4 count:

HCV/CMV/HSV:

X ray chest:

CT Chest:

MRI:

Histopathological examination:

MICROBIOLOGICAL EXAMINATION:

Direct examination: KOH & India ink

Gram stain

Culture :

Antimicrobial sensitivity pattern:

1. Disk diffusion:
2. MIC:

    By Broth dilution

    By Agar dilution

    By E test

APPENDIX IV- CONSENT FORM

STUDY TITLE :

A STUDY ON INVASIVE FUNGAL INFECTIONS AMONG IMMUNOCOMPROMISED PATIENTS IN A TERTIARY CARE HOSPITAL

Name: Date:

Age/Sex: IP.no:

I conform that I understand the purpose of the above study and I have the opportunity to ask questions. All my questions and doubts have been answered to my satisfaction. I understand that my participation in this study is voluntary and I am free to withdraw at any time without giving any reason. I understand that the investigator, regulatory authorities and ethical committees will not need my permission, to look at my health records both in respect to current study and any further research that conducted in relation to it. I also give my consent for my investigator to publish the data in any forum or journal.

I understand that my participation in the study will not affect my treatment. I have received information sheet regarding the study. I hereby consent to participate in the study "A STUDY ON INVASIVE FUNGAL INFECTIONS AMONG IMMUNOCOMPROMISED PATIENTS IN A TERTIARY CARE HOSPITAL" conducted at the Institute of Microbiology, Madras Medical college & Rajiv Gandhi Government General Hospital, Chennai-03

Date:

Place:

Signature/ Thumb impression of the patient

MASTER CHART

S.NO	AGE	SEX	SAMPLE	PERIOD OF CONDITION	RENAL TX	DM	CT	HIV	HCV	DIAGNOSIS	SYMPTOMS	HPE		ORGANISM	DEATH	D AMPHO	D ITRA	D FIU	MIC AMPHO	MIC ITRA	MIC FLU	MIC VORI	E TEST AMP	Agar dilution method AMP	AGAR DIL ITRA
1	60	Male	urine	7yrs	+	+				PUO/ UTI	fever,dysuria			C.tropicalis		S	S	S	S	S	S	R	S	S	S
2	72	Male	Blood	10yrs	+	+				sepsis	fever			C.glabrata		S	S	R	S	S	R	S	S	S	S
3	30	Male	pus	2yrs		+				brain abscess	headache,nausea,vomit	+	+	Asp.terreus		R	R		R	S		S	R	R	S
4	40	Male	Br.wash	3yrs		+				invasive aspergillosis	Fever and chills,hemoptysis,shortness of breath		+	Asp.flavus		S	S		S	S		S	S	S	S
5	60	Male	Br.wash	10yrs		+				consolidation	breathlessness, cough		+	Asp.terreus		R	S		R	S		S	R	R	S
6	43	Male	Aspirated FNAC	3yrs		+				infraorbital granuloma	orbital swelling,headache	+	+	Asp.flavus		S	S		S	S		S	S	S	S
7	34	Male	pus	2yrs	+				+	left thigh abscess		+	+	dematatious fungi									S	S	S
8	28	Male	BAL	2yrs	+				+	interstitial pneumonia	breathlessness, dry cough		+	Asp.fumigatus		S	S		S	S		S	S	S	S
9	47	Female	Br.wash	6yrs		+				pneumonia				Asp.fumigatus		SDD	S		S	S		S	S	S	S
10	36	Male	Br.wash	4yrs		+				pneumonia				Asp.fumigatus		R	S		S	S		S	S	S	S
11	54	Male	Br.wash	5yrs		+				Rt UL mass				Asp.fumigatus		S	S		S	S		S	S	S	S
12	22	Female	urine	4yrs	+					sepsis				C.parapsilosis		S	S	S	S	S	S	S	S	S	S
13	17	Female	urine	2yrs	+					PUO				C.tropicalis		S	S	S	S	S	S	R	S	S	S
14	45	Female	urine	5yrs		+				T2DM/sepsis				C.tropicalis		S	S	S	S	S	S	R	S	S	S
15	45	Male	Br.wash	6yrs		+				pneumonia	breathlessness, dry cough			Asp.fumigatus		S	S		S	S		S	S	S	S
16	29	Female	urine	6months	+					UTI	DIALYSIS			NG											
17	39	Male	BAL	4yrs		+				invasive aspergillosis	hemoptysis,shortness of breath			Asp.fumigatus		S	S		S	S		S	S	S	S
18	28	Female	IJV catheter tip	1 month	+					PUO				C.albicans		S	S	S	S	S	S	S	S	S	S
19	42	Female	Br.wash	3yrs	+				+	pneumonia	breathlessness, dry cough			Asp.fumigatus		S	R		S	S		S	S	S	S
20	57	Male	Br.wash	7yrs		+				pneumonia	breathlessness,dry cough			Asp.fumigatus		R	R		S	R		S	S	S	S
21	47	Male	Br.wash	7yrs		+				pneumonia	breathlessness,dry cough			Asp.fumigatus		S	SDD		S	S		S	S	S	S
22	47	Male	Br.wash	5yrs			+			CML & pneumonia	breathlessness,dry cough			Asp.flavus		S	S		S	S		S	S	S	S
23	42	Male	Blood	2yrs/ 1year(DM)	+	+			+	sepsis	Acute jaundice,Graft Failure, CMV			NG											
24	30	Male	orbital FNAC	5yrs	+	+				Rhinocerebral mucormycosis	orbital swelling,	+	+	Rhizopus oryzae		S	S		S	S		S	S	S	S
25	42	Female	urine	10yrs		+				UTI				C.albicans		S	S	S	S	S	S	S	S	S	S
26	30	Male	urine	6 months	+				+	UTI				NG											
27	23	Male	urine	6 months	+				+	UTI				NG											
28	26	Male	urine	1yr	+				+	UTI	Abd.pain,			NG											
29	42	Male	urine,drain,catheter	1 month	+				+					NG											
30	44	Male	urine,drain,catheter	1 month	+	+			+					NG											
31	40	Male	urine,drain,catheter	1 month	+						HSV			NG											
32	58	Male	FNAC debriment	8yrs		+				Rt Orbital cellulitis & fungal sinusitis		+	+	Absidia corymbifera		S	S		S	S		S	S	S	S
33	20	Male	urine,drain,catheter	1 month	+									NG											
34	27	Male	urine	4yrs	+				+	UTI	Graft dysfunction, HSV			NG											
35	14	Male	urine,drain,catheter	1 month	+									NG											
36	13	Female	urine,drain,catheter	1 month	+				+					NG											
37	26	Male	pus	4yrs	+					Left leg abscess				NG											
38	28	Female	urine	2yrs	+					UTI				NG											
39	33	Female	CSF	3yrs	+					meningitis	HSV			NG											
40	45	Male	BAL	7yrs		+				interstitial pneumonia		+		Asp.nidulans		S	S		S	S		S	S	S	S
41	40	Male	urine	2yrs	+					UTI	NEUTROPENIA			NG											
42	45	Male	Brain abscess	5yrs	+	+						+	+	Rhizopus oryzae	+	S	S		S	S		S	S	S	S
43	35	Female	sputum	5yrs		+						+		Exserohilum sp									S	S	S
44	30	Male	B.wash	5yrs		+				invasive aspergillosis	hemoptysis,shortness of breath	+		Asp.fumigatus		S	S		S	S		S	S	S	S
45	51	Male	BAL	7yrs		+				invasive aspergillosis	hemoptysis,shortness of breath	+		Asp.terreus		R	S		R	S		S	R	R	S
46	30	Female	B.wash	4yrs		+				consolidation	breathlessness			Curvaiaria lunaeta		SDD	S		S	S		S	S	S	S
47	28	Female	Urine	3yrs		+				UTI				C.tropicalis		S	S	R	S	S	S	R	S	S	S
48	40	Male	Urine	2yrs		+				UTI				NG											
49	20	Male	Urine	1yr		+				UTI				NG											
50	42	Male	urine,drain tube,catheter	1mon	+						NEUTROPENIA			NG											
51	26	Male	drain	10days	+									C.albicans		SDD	S	S	S	S	S	S	S	S	S
52	46	Male	urine/drain	1 month	+									NG											
53	35	Male	urine	2yrs	+					UTI				NG											
54	29	Male	urine	1mon	+					UTI				NG											
55	27	Male	urine	3yrs	+					UTI & graft failure	HSV			NG											
56	25	Male	Ascitic fluid	10yrs	+				+	Ascitis	Abd pain, abd distention			NG											
57	45	Male	pus	1yr	+					Genital ulcer	HSV			NG											
58	26	Male	Urine	2yrs	+					UTI	NEUTROPENIA			NG											
59	42	Male	Blood	14yrs	+				+	Graft failure				NG											
60	24	Male	Blood	9months	+					PUO	Fever, jaundice, HSV			NG											
61	60	Female	Br.wash	17yrs			+			Ca oesophagus				NG											
62	40	Male	Ascitic fluid	2yrs			+			Ca head of pancreas				NG											
63	60	Male	Br.wash	19yrs			+			Ca oesophagus				NG											
64	75	Male	Br.wash	20yrs			+			Ca oesophagus				NG											
65	33	Male	pl.fluid	2yrs			+			Pleural malignancy				NG											
66	38	Female	Gastric lavage	3yrs			+			Gastric ca				C.albicans		S	S	S	S	S	S	S	S	S	S
67	68	Male	Br.wash	18yrs			+			Ca lung				Asp.flavus		SDD	R		S	S		S	S	S	S
68	48	Male	Br.wash	14yrs			+			Ca lung & secondaries in neck				NG											
69	40	Female	urine	3yrs		+				T2DM/UTI				NG											
70	20	Female	Urine	2yrs		+				T2DM/UTI				NG											
71	37	Female	Urine	3yrs		+				T2DM/UTI				NG											
72	38	Female	urine	4yrs		+				T2DM/UTI				C.parapsilosis		S	S	S	S	S	S	S	S	S	S
73	40	Female	urine	2yrs		+				T2DM/UTI				NG											
74	46	Female	urine	3yrs		+				T2DM/UTI				NG											
75	52	Female	urine	2yrs		+				T2DM/UTI				NG											
76	35	Female	urine	2yrs		+				T2DM/UTI				NG											
77	35	Female	urine	1yr		+				T2DM/UTI				NG											
78	55	Male	urine	4yrs		+				T2DM/UTI				NG											
79	44	Female	urine	3yrs		+				T2DM/UTI				C.albicans		S	S	S	S	S	S	S	S	S	S
80	45	Female	urine	5yrs		+				T2DM/UTI				NG											
81	31	Male	urine	2yrs		+				T2DM/UTI				NG											

82	51	Male	urine	5yrs		+				T2DM/UTI				NG														
83	55	Male	urine	4yrs		+				T2DM/UTI				NG														
84	45	Male	urine	3yrs		+				T2DM/UTI				NG														
85	35	Male	urine	2yrs		+				T2DM/UTI				NG														
86	50	Male	urine	5yrs		+				T2DM/PUO	PUO			C.albicans		S	S	SDD	S	S	S	S	S	S	S	
87	55	Male	urine	7yrs		+				T2DM/UTI				C.albicans		S	S	S	S	S	S	S	S	S	S	
88	50	Male	urine	5yrs		+				T2DM/UTI				NG														
89	23	Male	urine	3yrs		+				T1DM/UTI				NG														
90	39	Male	urine	3yrs		+				T2DM/UTI				NG														
91	45	Female	urine	6yrs		+				T2DM/UTI				NG														
92	18	Male	urine	2yrs		+				T1DM/UTI				NG														
93	33	Male	urine	3yrs		+				T2DM/UTI				NG														
94	63	Female	urine	12yrs		+				T2DM/UTI				C.albicans		R	S	R	S	S	S	S	S	S	S	
95	50	Male	urine	5yrs		+				T2DM/UTI				NG														
96	39	Male	pus	3yrs		+				T2DM & pneumonia				Asp.flavus		R	R		S	S		S	S	S	S	
97	68	Male	pl.fluid	15yrs		+				Rt loculated empyema				Asp. terreus		R	S		R	S		S	R	R	S	
98	17	Male	urine,drain,catheter	1 month	+						DIALYSIS			NG													
99	45	Female	pus	10yrs		+				Rt thigh abscess- lateral aspect				C.albicans		S	S	S	S	S	S	S	S	S	S	
100	20	Male	sputum	5yrs			+			AML			+	Fusarium sp		R	R		R	R		S	R	R	R	
101	50	Male	Ascitic fluid	10yrs		+	+			Peritoneal carcinomatation with liver metastasis		+		Asp.flavus		S	S		S	S		S	S	S	S	
102	39	Female	urine	5yrs	+					UTI	fever,dysuria			C.albicans		S	S	S	S	S	S	S	S	S	S	
103	55	Female	urine	7yrs		+				UTI				C.glabrata		S	S	R	S	S	R	S	S	S	S	
104	61	Female	urine	12yrs		+				UTI				C.albicans		S	S	S	S	S	S	S	S	S	S	
105	22	Female	urine	4yrs		+				UTI				C.albicans		S	S	SDD	S	S	S	S	S	S	S	
106	53	Male	Br.wash	8yrs		+				Lt LL consolidation				Penicillium sp		S	S		S	S		S	S	S	S	
107	56	Male	Br.wash	12yrs		+				Rt lung mass				Penicillium sp		S	SDD		S	S		S	S	S	S	
108	47	Male	Br.wash	7yrs		+				invasive aspergillosis				Asp.fumigatus		S	S		S	S		S	S	S	S	
109	36	Male	drain	3yrs	+					ADD				C.glabrata		S	S	R	S	S	R	S	S	S	S	
110	45	Male	Br.wash	7yrs		+				Rt LL consolidation				NG														
111	42	Male	sputum	8yrs		+				T2DM/fungal ball				Asp.fumigatus		S	S		S	S		S	S	S	S	
112	38	Male	pus	3yrs				+		liver abscess				NG														
113	42	Male	Br.wash	5yrs			+			Ca lung			+	Penicillium sp		S	S		S	S		S	S	S	S	
114	47	Male	pus	9yrs			+			Ca lung & multiple abscess all over body				C.albicans		S	S	S	S	S	S	S	S	S	S	
115	64	Male	Gastric lavage	10yrs			+			Ca oesophagus				C.albicans		S	S	S	S	S	S	S	S	S	S	
116	61	Male	sputum	5yrs			+			Mediastinal mass				Asp.fumigatus		S	SDD		S	S		S	S	S	S	
117	36	Male	FNAC debriment	6yrs		+				Cavernous sinus thrombus	facial puffiness,R nasal block	+	+	Rhizopus oryzae	+	S	S		S	S		S	S	S	S	
118	23	Female	sputum	10yrs			+			AML& Rt basal consolidation		+		Asp.fumigatus	+	R	R		R	R		S	S	S	S	
119	35	Male	urine	5yrs	+					UTI				C.tropicalis		S	S	S	S	S	S	R	S	S	R	
120	35	Male	urine,drain,catheter	1mon	+					CMV	DIALYSIS			NG														
121	32	Male	urine,drain,catheter	1mon	+				+					NG														
122	42	Male	urine,drain,catheter	1mon	+					CMV	NEUTROPENIA			NG														
123	25	Female	urine	7yrs	+				+					NG														
124	27	Male	urine,drain,catheter	1mon	+					CMV	DIALYSIS			NG														
125	22	Male	urine	1yr	+					UTI, CMV	DIALYSIS			NG														
126	24	Female	urine	1yr	+					UTI	NEUTROPENIA			NG														
127	41	Male	urine	1yr	+					UTI	NEUTROPENIA			NG														
128	28	Male	urine,drain,catheter	1mon	+						DIALYSIS			NG														
129	25	Female	urine	1yr	+					UTI, CMV	NEUTROPENIA			NG														
130	33	Male	urine,drain,catheter	1mon	+				+					NG														
131	30	Male	urine,drain,catheter	1mon	+						NEUTROPENIA			NG														
132	27	Female	urine	2yrs	+					UTI	CMV			NG														
133	25	Female	urine,drain,catheter	1mon	+						DIALYSIS			NG														
134	37	Male	urine,drain,catheter	1mon	+				+					NG														
135	30	Female	drain	3yrs	+					ADD, CMV	NEUTROPENIA			NG														
136	33	Male	urine,drain,catheter	1mon	+						DIALYSIS			NG														
137	30	Male	urine,drain,catheter	1mon	+						CMV			NG														
138	42	Male	Blood	7yrs		+				sepsis				C.glabrata		S	S	R	S	S	R	S	S	S	S	
139	55	Male	sputum	15yrs			+			NHL				NG														
140	52	Male	pl.fluid	5yrs			+			Ant.mediastinal mass				NG														
141	32	Male	pl.fluid	2yrs			+			Malignant mesothelioma				NG														
142	45	Male	Ascitic fluid	3yrs			+			Ca stomach with liver metastasis				NG														
143	59	Male	sputum	9yrs			+			Ca lung		+		Asp.fumigatus		S	S		S	S		S	S	S	S	
144	30	Male	sputum	5yrs		+				invasive aspergillosis		+		Asp.niger		S	S		S	S		S	S	S	S	
145	64	Male	Br.wash	8yrs		+				invasive aspergillosis				Asp.fumigatus		SDD	S		S	S		S	S	S	S	
146	65	Male	pl.fluid	15yrs		+				invasive aspergillosis				Asp.fumigatus		S	S		S	S		S	S	S	S	
147	46	Male	sputum	6yrs			+			MDS & invasive aspergillosis		+		Asp.flavus		S	SDD		S	S		S	S	S	S	
148	52	Male	sputum	10yrs			+			MDS & Aspergilloma				Asp.fumigatus		S	S		S	S		S	S	S	S	
149	55	Female	Br.wash	10yrs		+	+			Ca lung			+	Asp.flavus		S	S		S	S		S	S	S	S	
150	38	Male	Blood	5yrs		+				sepsis				C.albicans		S	S	S	S	S	S	S	S	S	S	
151	48	Male	Br.wash	7yrs			+			Ca lung			+	Asp.fumigatus		SDD	S		S	S		S	S	S	S	
152	20	Male	blood	6yrs			+			AML				C.albicans	+	R	R	R	R	R	R	S	R	R	S	
153	62	Male	sputum	7yrs			+			Ca sigmoid colon				NG														
154	38	Male	pl.fluid	8yrs			+			Ca lung				NG														
155	40	Male	sputum	4yrs			+			Ca stomach with liver metastasis				NG														
156	62	Male	sputum	12yrs			+			Ca ceacum				NG														
157	14	Male	sputum	2yrs			+			Rtfemur osteosarcoma				NG														
158	69	Male	sputum	15yrs			+			Ca sigmoid colon				NG														
159	15	Male	pl.fluid	1yr			+			Lt tibial osteosarcoma	pleural effusion			NG														
160	60	Male	sputum	10yrs			+			Ca lung				NG														
161	31	Male	Gastric lavage	4yrs	+					oesophageal candidiasis	dysphagia, NEUTROPENIA			NG														
162	51	Male	urine	3yrs	+					UTI				C.albicans		SDD	SDD	SDD	S	S	S	S	S	S	S	
163	62	Male	sputum	12yrs			+			Ca lung				NG														
164	45	Male	sputum	2yrs			+			Ca gall bladder & liver metastasis				NG														
165	30	Male	ascitic fluid	3yrs			+			Ca ascending colon				NG														
166	32	Male	pl.fluid	5yrs			+			NHL				NG														
167	30	Male	sputum	5yrs	+					pneumonia	CMV			NG														
168	42	Male	sputum	7yrs			+			MDS				NG														
169	31	Male	Gastric lavage	6yrs	+					esophageal candidiasis	dysphagia			C.albicans		S	S	S	S	S	S	S	S	S	S	

170	33	Male	urine	6yrs	+					UTI	CMV			NG											
171	40	Male	Gastric lavage	7yrs				+		esophageal candidiasis		+		C.albicans		S	S	S	S	S	S	S	S	S	S
172	28	Male	CSF	4yrs				+		meningitis				NG											
173	45	Male	CSF	5yrs				+		meningitis				NG											
174	35	Male	palatal scraping	3yrs				+		mucormycosis				NG											
175	27	Female	CSF	3yrs				+		meningitis				NG											
176	45	Male	CSF	7yrs				+		meningitis		+		Cryptococcal sp		S		S	S		S	S	S	S	
177	20	Female	CSF	2yrs				+		meningitis				NG											
178	45	Male	CSF	6yrs				+		meningitis				NG											
179	55	Male	CSF	8yrs				+		meningitis				NG											
180	39	Male	CSF	6yrs				+		meningitis		+		Cryptococcal sp		S		S	S		S	S	S	S	
181	70	Male	CSF	12yrs				+		meningitis				NG											
182	14	Female	CSF	2yrs				+		meningitis				NG											
183	56	Female	palatal scraping	6yrs				+		mucormycosis				NG											
184	60	Female	Gastric lavage	12yrs				+		esophageal candidiasis				NG											
185	32	Female	CSF	4yrs				+		meningitis				NG											
186	38	Female	CSF	5yrs				+		meningitis		+		Cryptococcal sp		S		S	S		S	S	S	S	
187	56	Male	CSF	6yrs				+		meningitis				NG											
188	35	Female	CSF	5yrs				+		meningitis				NG											
189	33	Male	CSF	3yrs				+		meningitis		+		Cryptococcal sp	+	S		S	S		S	S	S	S	
190	34	Male	CSF	3yrs				+		meningitis				NG											
191	17	Male	CSF	5yrs				+		meningitis				NG											
192	21	Male	sputum	5yrs			+			AML & PNEUMONIA				NG											
193	21	Male	sputum	6yrs			+			AML RT ul consolidation				NG											
194	35	Male	CSF	5yrs				+		meningitis				NG											
195	22	Female	graft abscess		+					PUO	HSV			NG											
196	40	Female	sputum	7yrs			+			AML & PNEUMONIA				NG											
197	21	Female	sputum	5yrs			+			ALL		+	+	Fusarium sp		R	R		R	R		S	R	R	S
198	17	Male	sputum	2yrs			+			CML & pneumonia				NG											
199	55	Female	BAL	8yrs			+			MDS & pneumonia				NG											
200	70	Male	FNAC debriment	15yrs		+				Mucormycosis of Rt palate				NG											